A rational approach to the development of future generation processes for lipoprotein VLP vaccine candidates

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By

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Declaration

The author confirms that this thesis presents work which is the author's own except in the areas listed below.

- **Triton X-100 concentration screen** for the evaluation of the impact of the detergent on HBsAg recovery and activity (Chapter 4). This study was performed as part of an MEng research project with S. Feiz (MEng Biochemical Engineering, UCL, 2007).
- **Ultra scale-down ultrafiltration (UF) studies** comparing ultrafiltration process performance for process streams generated using different concentrations of Triton X-100 detergent. This study was performed in collaboration with G. Ma using the USD device he developed.
- **Extraction and analysis of nucleic acids** for comparing the purification performance of the conventional and selective recovery operations (Chapter 6). This analysis was performed with assistance from S. Yau.
- **Hydrophobic interaction chromatography (HIC) challenge** for assessing the benefits of the optimised selective product recovery methodology on downstream chromatography (Chapter 6). This study was performed in collaboration with J. Jing.

Abstract

Lipoprotein VLPs, known also as lipid-envelope VLPs, are expressed intracellularly in yeast, localised on the host endoplasmic reticulum (ER). The main challenge in production lies in the complexity of the recovery and purification process. Using the Hepatitis B surface antigen (HBsAg) as the VLP model, strategies for exploiting the full potential of primary recovery operations to raise the overall process efficiency and throughput were investigated.

Detergent-mediated liberation of HBsAg from the host endoplasmic reticulum is a critical step which influences product quality and yield and defines the characteristics of the resulting process stream. Screening studies established that Triton X-100 gave superior performance if concentrations were maintained within the range of 0.2% v/v to 0.5% v/v. Concentrations above this threshold led to HBsAg delipidation and loss of antigenicity. Increased levels of co-liberated host protein and of lipid contamination which resulted in poorer ultrafiltration performance were also observed.

PEG and ammonium sulphate precipitations were investigated to reduce the level of host protein and lipid contaminants. In each case, although significant product enrichment was achieved, product loss of up to 50% was incurred owing to the difficulty of PEG precipitate recovery and resolubilisation and the lack of selectivity of the ammonium sulphate agent. A novel selective product recovery methodology was developed in which an additional centrifugation step was introduced post-cell disruption but prior to the addition of detergent. HBsAg associated with the ER was pelleted allowing bulk cytosolic contaminants in the supernatant to be eliminated. This approach was further enhanced by the use of moderate homogenisation pressures in the region of 400 bar. Recovery of active HBsAg improved by ~20% under lower shear conditions and a further ~40% reduction in the level of host lipid contaminants was observed. The benefits of the proposed process on a downstream chromatography step include better product capture and improved step yield.

The scalability of the selective recovery method relies on the dewatering and clarification performance. A CARR tubular type centrifuge was identified to be the most suitable equipment type for this purpose. Ultra scale-down centrifugation experiments were employed to predict suitable operating conditions for a pilot-scale process which was subsequently carried out and successfully verified that the selective recovery methodology remained feasible upon scale-up.

Overall, this study has demonstrated how the adoption of a rational approach to the design of primary purification operations can lead to significant improvements of downstream purification performance.

Acknowledgement

This thesis represents the culmination of a 3-year long journey undulated with research highs and lows. This journey would have been impossible without the supervision from Prof Nigel Titchener-Hooker. I wish to thank Nigel for his mentoring, unwavering support and patience in ensuring I stayed on course.

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In the course of this PhD, I have had the privilege of working alongside many others at UCL. I wish to thank Dr Daniel Bracewell and Dr Balasundaram Bangaru for their ideas and input on VLP research. I would like to extend my sincere gratitude to Jing Jin and Claire Burden who have been incredibly supportive and helpful as part of the VLP team. I wish to acknowledge Dr Gareth Mannall and Mr Ian Buchanan for the training and their assistance in the pilot plant. I am also grateful to Sahar Feiz and Guijun Ma for their assistance in specific areas of the research.

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Last but not least, I am grateful to my family and friends for all their love and support.

To him whose strength is made perfect in my weakness.

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Chapter 1

Introduction

1.1 Background and motivation to the project

This study investigates new strategies for improving the primary purification process for future generation lipid envelope virus-like particle (VLP) vaccines using the recombinant hepatitis B surface antigen (HBsAg) expressed in *Saccharomyces cerevisiae* as the VLP model. The findings presented in this study would benefit process design for novel VLPs bearing similar lipid-envelope structures and chimeric VLPs constructed on a HBsAg platform.

VLPs are assemblies of virus capsid or envelope proteins mimicking the native viral structure but in the absence of the viral genome. In the form of VLPs, vaccine proteins are presented in their natural conformations and trigger powerful immune responses. Lacking viral DNA or RNA, VLPs offer a safe and effective vaccination approach compared to traditional vaccines which suffer from risks of viral reversion, recombination or re-assortment. VLP vaccines against hepatitis B and human pappilomavirus (HPV) have been licenced while others are at different stages of the development pipeline (Grgacic and Anderson, 2006).

Virus-like particles can be categorised broadly as simple non-envelope VLPs and lipid-envelope VLPs (Noad and Roy, 2003). The lipid-envelope subclass is of specific interest in this study since viruses causing many diseases including HIV,

dengue fever and hepatits B and C exist in envelope structures in the natural state (Bisht *et al.*, 2001; Yao *et al.*, 2000; Baumert *et al.*, 1998; Betenbaugh *et al.*, 1995). The lipid environment is important for the correct folding and presentation of the antibody neutralising epitopes (Greco *et al.*, 2007) however the re-creation of the virion lipid-envelope is often difficult (Grunder *et al.*, 2002). The lipoprotein structure of the pre-established HBsAg particle can be used as a platform for the display of foreign epitopes which are otherwise difficult to reconstruct *de novo* (Netter *et al.*, 2001; Bisht *et al.*, 2001; Michel *et al.*, 2007; Greco *et al.*, 2007).

In spite of the obvious merits of VLP vaccines, it is recognised that their potential has yet to be fully realised (Pattenden *et al.*, 2005). One of the main reasons for this is the higher costs of production (Boisgerault *et al.*, 2002). VLP vaccines require sophisticated and expensive technologies for production and characterisation compared to those in the production of live attenuated vaccines. The stricter regulatory oversight (Buckland, 2005) derived from the fact that vaccines are to be administered to healthy individuals impact every step in the manufacturing cycle and further contribute to the high costs of production. From a manufacturing perspective, changes in the VLP microenvironment during processing could have significant impact on its biophysical attributes (Shi *et al.*, 2005) and the process of producing the VLPs has been described as what defines the end product (Buckland, 2005).

This project aims to address some of the challenges discussed above from the perspective of the VLP manufacturing process. It is well recognised that downstream processing is the main cost contributor in the manufacturing of VLPs. It is thus not surprising that process development efforts are centred on these operations. Primary purification which could be equally critical in setting the backdrop for downstream operations and in determining the overall process performance is often overlooked. It is

the target of this thesis to address some of the disparities in process design. Specific emphases in this study are on improving the performance of primary purification and optimising primary and downstream process interactions so as to increase overall process efficiency and throughput.

Since the process defines the product, it is vital that the intrinsic physiochemical properties of the VLPs are taken into account at all stages of processing. Specifically for lipid-envelope VLPs such as the HBsAg, the detergent step which facilitates VLP recovery from associated membranous organelles could present a hidden challenge. Although high yield is important, equally so is the VLP potency. Hence, it is critical that the use of detergent does not have negative consequences on the conformation or composition of the VLP. High concentrations of detergent would favour VLP recovery based on reaction kinetics. Excess detergent, however, could detrimentally affect the VLP lipid constituents leading to reduced immunogenicity (Gavilanes *et al.*, 1990). In this respect, the benefits of improved VLP recovery need to be traded-off with the risks of VLP activity loss when identifying the optimal operating range for the detergent step.

Like most protein recovery operations, the cost in VLP production is dominated by the expensive and low throughput chromatographic operations (Przybycien *et al.*, 2004). Although alternatives to chromatography such as membrane filtration, aqueous two-phase extractions, crystallisation and membrane chromatography have been proposed, it is unlikely that the use of chromatography can be excluded entirely. Nevertheless, the ability to reduce the reliance on chromatography and to improve its throughput is highly attractive (Leser and Asenjo, 1992; Tsoka *et al.*, 2000; Przybycien *et al.*, 2004). One means of achieving this is by maximising the potentials of the initial recovery and purification stage to attain significant levels of reduction in contaminants such as host lipids and proteins, higher product enrichment and lower processing

volumes entering the higher resolution downstream operations. This study proposes a two-tier strategy for this purpose: (1) by reducing the levels of host lipids and proteins which are co-released into the process stream during detergent-mediated VLP liberation, and (2) by selectively removing lipid and protein contaminants in the process stream via the introduction of a bulk separation or fractionation step.

Although the bulk of contaminants from *S.cerevisiae* originate from the cell cytosol, membranous organelles such as the endoplasmic reticulum (ER), golgi bodies and plasma membrane are also rich in proteins and lipids. It is likely that during the VLP liberation step by detergent, significant proportions of membrane associated lipids and proteins are co-released. VLP yield and the contamination levels would both vary as a function of the detergent concentration but could have different rates of release. Optimisation studies could be performed to identify a suitable operating range for the detergent step where high yields of VLP are achieved without the expense of high contamination levels. The release rates of VLPs and contaminants during the detergent step could also be influenced by the degree of cell disruption and size of debris formed. Extensive cell disruption could improve VLP recovery however the smaller debris particulates formed would create larger surface area for the extraction of membrane lipids and proteins by detergent. Likewise, a trade-off study would be useful here to determine the suitable operating range.

Different approaches are possible for improving the effectiveness of eliminating host-derived contaminants during primary purification. Precipitation using polyethylene glycol (PEG) or ammonium sulphate is commonly employed to achieve selective fractionation of VLP prior to downstream purification (Tsoka *et al.*, 2000; Huang *et al.*, 2007, Wijnendaele *et al.*, 1987). Although the use of PEG and ammonium sulphate precipitation has been demonstrated in the purification of the blood-derived HBsAg

vaccine (Einarsson, 1983; Peterson *et al.*, 1981), their application for the recombinantly expressed HBsAg vaccine is limited. Since recombinantly expressed VLPs are not completely identical to the blood-derived ones, the precipitation conditions and performance may differ.

Unique VLP compartmentalisation characteristics of membrane-envelope VLPs such as the localisation of HBsAg on the yeast endoplasmic reticulum (ER) can also be exploited for the purpose of selective product release. For example, following cell disruption but prior to VLP liberation by detergent, a solids-liquid separation step by centrifugation could be employed to sediment VLPs which are still compartmentalised within the associated membranous organelle allowing the supernatant containing bulk contaminants originating from the cytosol to be eliminated. When the solids fraction is later treated with detergent, the VLP product would be released into a process stream with minimal cytosolic contaminants. Application of such selective product release for VLP purification has been demonstrated at a laboratory scale (Chi *et al.*, 1994) but the study had been limited to the protein class of contaminants.

Scale-up considerations are crucial from the onset of process design since once a process is in place, any corrective actions would be expensive and could take years for approval from the regulatory authorities (Buckland, 2005). Process development efforts at laboratory scale can lead to inefficient process design as some of the putative processes may indeed prove difficult or costly to scale up and not be amenable to late-stage bioprocess modifications (Pattenden *et al.*, 2005). Scale-down bioprocess technologies are highly attractive in this light as they allow the development and prediction of scalable processes with minimal developmental time and costs (Titchener-Hooker *et al.*, 2008). Successful scale-down of commonly employed downstream operations such as homogenisation (Hetherington *et al.*, 1971), centrifugation (Higgins

et al., 1978), precipitation (Boychyn *et al.*, 2000) and chromatography (Janson and Dunhill, 1978) have been demonstrated. These scale-down techniques are invaluable here to ensure that the primary purification processes developed at laboratory scale are equally effective upon scale-up (Boychyn *et al.*, 2000; Pattenden *et al.*, 2005).

This thesis describes how a systemic approach can be adopted in designing an efficient primary purification process. This would take into account the different factors discussed for the development of an improved primary purification process for future generation lipid-envelope VLP vaccines. The strategy employed here places great significance on understanding the expression system and the physiochemical attributes of VLPs, as well as the process interactions and engineering factors which together determine process performance. The next section provides the background to virus-like particles and the challenges facing its production and purification while the specific aims of the project are detailed in **Section 1.6**.

1.2 Introduction to virus-like particles

Virus-like particles (VLPs) are recombinant virus capsid or enveloped proteins (Grgacic and Anderson, 2006) self-assembled in a host cell in the absence of viral DNA and RNA genome (Noad and Roy, 2003). The VLPs consist of highly repetitive and ordered structures mimicking the native structure and antigenic properties of the parental virus which can trigger potent humoral and cellular responses (Boisgerault *et al.*, 2002). The VLPs are devoid of viral genetic material which ensures that any recipient bears no risks of infection. From this perspective, VLPs hold a major advantage over traditional vaccines based on inactivated or attenuated viruses which suffer from risks of viral reversion, recombination or re-assortment (Noad and Roy,

2003). Additional benefits of VLP vaccines include higher efficiency in eliciting a protective response and lower dosage requirements relative to subunit vaccines, in particular, which are based on purified antigen rather than the whole organism.

There is increasing interest in the production of recombinant VLPs as vaccine candidates. The hepatitis B VLP, Recombivax HB®, from Merck which was the first of such vaccines, licensed for human use in 1984 (Hilleman, 2001; Hilleman, 2003; McAleer *et al.*, 1984; Valenzuela *et al.*, 1982). Recently, VLP vaccines against the human pappilomavirus (HPV), Gardasil® and Cerevix® by Merck and GlaxoSmithKline (GSK), respectively were licenced (Andrawiss, 2007; Schiller and Lowly, 2006). VLP based vaccines for over 30 viral diseases affecting humans and animals have been developed hitherto and are at different stages of the development pipeline (Noad and Roy, 2003; Grargic and Anderson, 2006).

VLPs can be broadly categorised as simple non-enveloped VLPs and lipid-envelope VLPs. The HPV vaccine is an example of a simple non-enveloped VLP. Such VLPs are assembled from one or two major capsid proteins of viruses which are relatively simple in structure and do not have a lipid envelope. The hepatitis B vaccine is an example of the latter category. The VLPs are assembled as envelope proteins bud from cellular compartments such as the ER, plasma membrane of lipid rafts taking with them cellular lipid organised to form lipoprotein particles (Grargic and Anderson, 2006). Production of lipid-envelope VLPs has been reported to be the more challenging of the two. Additional technical issues for consideration for lipid-envelope VLPs include the choice of the expression system which would directly affect the envelope which is host-derived (Betenbaugh *et al.*, 1995; Buonaguro *et al.*, 2005) and the need to prevent interference of the envelope structure with the presentation of the immunogenic viral components (Yamshchikov *et al.*, 1995; Baumert *et al*, 1998).

In addition to the direct immunogenicity of VLPs, they have also been shown to be efficient in stimulating cellular and humoral responses (Schirmbeck *et al.*, 1996; Paliard *et al.*, 2000; Murata *et al.*, 2003) making them excellent candidates as carrier molecules or presentation platforms for the delivery of epitopes targeted at other diseases. The formation of chimeric VLPs as such is possible through the genetic engineering of the VLP gene sequence to include genes of the foreign vaccine epitope and fusion proteins so that these proteins are co-expressed and assembled onto the VLP during synthesis. The development of chimeric VLPs significantly expands the niche of VLPs in preventative medicines beyond disease conditions in which VLPs are naturally formed (Grgacic and Anderson, 2006; Noad and Roy, 2003). The same principles may also be applied in the production of multivalent vaccines targeting more than one unrelated diseases simultaneously (Netter *et al.*, 2001; Michel *et al.*, 2007). [The full account of the range of chimeric vaccines, vaccine status in the developmental pipeline, the advantages and disadvantages and technical considerations are outlined elsewhere (Grgacic and Anderson, 2006; Boisgerault *et al.*, 2002).] Another possibility for future use of VLPs lies in the ability of VLPs to assemble and disassemble when induced by changes in the chemical environment. This feature could be exploited for carrying either foreign genes or chemical agents into cells (Goldmann *et al.*, 1999; Goldmann *et al.*, 2000).

It has been reported that in spite of the unique properties of VLPs, the number of licensed VLP vaccines remains low for the reason that sophisticated and expensive technologies are required in manufacture as compared to the lower costs incurred in the production of live attenuated vaccines (Pattenden *et al.*, 2005; Boisgerault *et al.*, 2002). The higher production costs for VLP vaccines would translate to significantly more expensive vaccination programmes and thus are viewed less favourably by health care

providers. Specific challenges in VLP process development and the associated regulatory constraints have been reviewed (Buckland, 2005). To achieve lower costs in VLP manufacturing and rapid delivery to the market, bioprocess manufacturing research is as critical as the product development itself (Pattenden *et al.*, 2005). As discussed previously, it is the focus of this project to study various means of improving the bioprocessing of VLPs to complement the enhanced capacity for the development of novel VLP vaccines.

1.3 Introduction to the hepatitis B disease and the hepatitis B surface antigen (HBsAg) vaccine

1.3.1 Hepatitis B disease

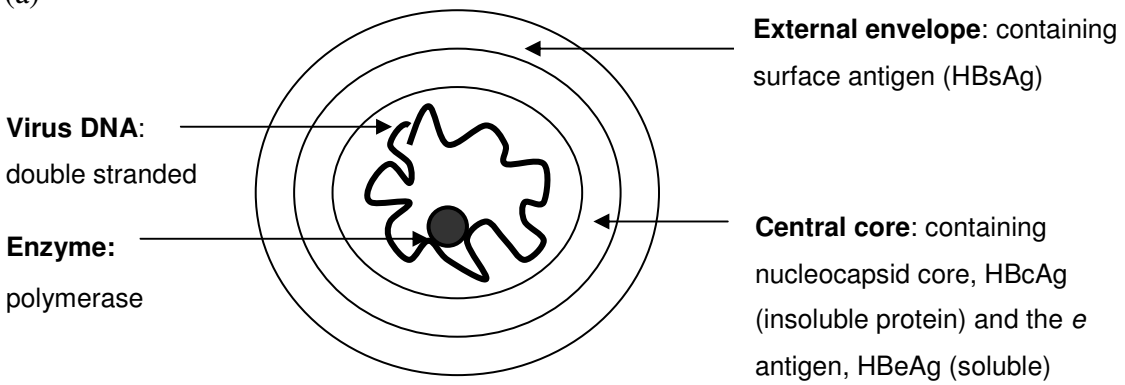
Hepatitis B is the most serious liver infection in the world and can lead to liver failure, cirrhosis or cancer of the liver (Hepatitis B Foundation). It is often characterised by jaundice, abdominal pain, liver enlargement and fever. According to the WHO, it is estimated that 400 million people worldwide are already chronically infected with hepatitis B and this disease leads to over 1 million deaths per year. This disease is transmitted by blood and body fluids, frequently during sexual intercourse. Treatment, is generally ineffective (Hepatitis B Foundation) and thus prevention and vaccination remain the best alternatives for this disease.

1.3.2 Hepatitis B virus

Hepatitis B is caused by the hepatitis B virus (HBV), an envelope virus containing a partially double stranded, circular DNA genome and classified within the family hepadnavirus (Shepard *et al.*, 2006). The virus interferes with the function of the liver in replicating hepatocytes causing the immune system to be activated leading to liver inflammation.

The Hepatitis B virus is a spherical particle with a diameter of 42 – 47 nm and circulates in the blood in concentrations as high as 10^8 virions per mL (Shepard *et al.*, 2006). The particles are composed of an outer shell or envelope, which consists of proteins known as HBs or surface proteins. These self-assembling units form non-infectious spherical or tubular particles (Robinson and Lutwick, 1976) containing the surface antigens (HBsAg) which are responsible for eliciting the immune response. The HBsAg particles contain only envelope glycoproteins and host-derived lipids and outnumber virions by 1000:1 to 10,000:1 (Ganem and Prince, 2004). The surface coat surrounds an inner protein shell, composed of HBc proteins. Finally, the viral DNA and enzyme DNA polymerase can be found in the inner most region of the particle. A schematic of the cross section of the hepatitis B virus is as shown in **Figure 1.1(a)** while **Figure 1.1(b)** shows the 3D structure of the virus resolved from X-ray crystallography studies (Srgo, 2004).

(a)



(b)

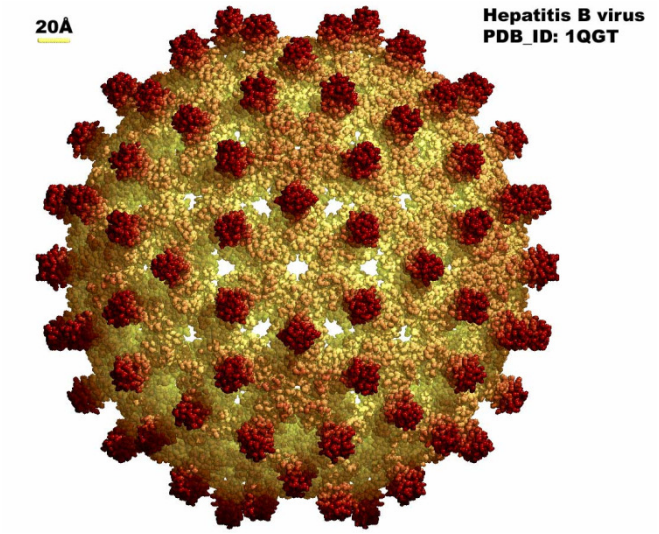


Figure 1.1*: (a) shows a schematic of the cross section of a hepatitis B virus. (b) shows the structure of the hepatitis B virus as solved by x-ray crystallography, image by Sgro (2004) (taken from http://www.virology.wisc.edu/virusworld/covers.php).*

When the virus invades the host, the virus binds to the cell surface and penetrates with the help of its envelope proteins. The virus is not degraded inside the cell but is transported to the nucleus (Ganem and Prince, 2004). The partially circular DNA of HBV is initially made into covalently closed circular DNA (Tuttleman *et al.*, 1986), which functions as a template for RNA synthesis. The RNA is then reverse transcribed into open circular DNA which is packaged into viral envelopes in the ER

and transported out of the cell, potentially leading to a new round on infection. Viral replication continues inside the host's liver cells.

1.3.3 Brief history of treatment for the Hepatitis B disease

Hepatitis B vaccines have been available since the early 1980s. The first vaccine (HEPTAVAX®), licensed in 1981, was a subunit vaccine isolated from the blood of hepatitis B infected donors (Prince, 1982). In hepatitis B patients suffering from acute infection and in hepatitis B carriers, 22nm particles containing HBV surface antigen subunits of the virus are found expressed in copious amounts. These particles themselves are not infectious but are effective in stimulating a strong immune response (Dekleva, 1999). Since these particles are derived from blood, extensive purification is critical to ensure that the final product exceeds 99% purity and is free from extraneous infectious viruses.

Historically, there have been many limitations in the manufacture of plasma-derived hepatitis B vaccines. Primarily, a constant limitation exists in the supply of human plasma. Due to the intensity of the manufacturing process, more than 1 year is required to produce just one batch (Dekleva, 1999). During the 1980s there was also growing concerns about AIDS infection through blood derived products (Francis *et al.*, 1986) which led to pressures to develop a recombinant vaccine for human use. RECOMBIVAX HB® from Merck was the first recombinant hepatitis B VLP vaccine. GlaxoSmithKline (GSK) has produced a recombinant hepatitis B vaccine, ENGERIX B®, which is also currently in the market. In addition, there is now a vaccine for both hepatitis A and B together, known as TWINRIX®, which is bivalent in nature, containing antigenic components used in producing HAVRIX® (Hepatitis A inactivated

vaccine) and ENGERIX-B® (Hepatitis B, recombinant vaccine), by GlaxoSmithKline (GSK).

1.3.4 Hepatitis B Surface Antigen (HBsAg) VLP vaccine

The first commercially manufactured recombinant hepatitis B VLP licensed in 1984 (Hilleman, 2001; Hilleman, 2003; McAleer *et al.*, 1984; Valenzuela *et al.*, 1982) was cloned and expressed in *S. cerevisiae* (McAleer *et al.*, 1984; Miyanohara *et al.*, 1983). The VLP is assembled from up to 100 copies of the hepatitis B surface antigen monomer and host-derived lipids forming highly immunogenic spherical particles measuring ~ 22nm in diameter. A cross-sectional representation of a HBsAg particle is shown in **Figure 1.2 (a)**. The VLP is widely referred to as the hepatitis B surface antigen (HBsAg) after its origins. Comparison of the HBsAg produced by recombinant yeast to HBsAg of human blood plasma has been reported (Yamaguchi *et al.*, 1998).

In *S. cerevisiae*, the assembly of the recombinant HBsAg particles is initiated by the insertion of the hydrophobic segments of the HBsAg monomer into the endoplasmic reticulum (ER) (Zhao *et al.*, 2006) which then bud into the lumen of the ER as 20-nm lipoprotein particles using host-dervied machinery and lipids (Eble *et al.*, 1986). In humans, the HBsAg maturation process will proceed through the Golgi and the HBsAg particles are subsequently secreted. In yeast cells however, due to the lack of protein transport machinery (Biemans *et al.*, 1992), the HBsAg particles are permanently localised in the ER.

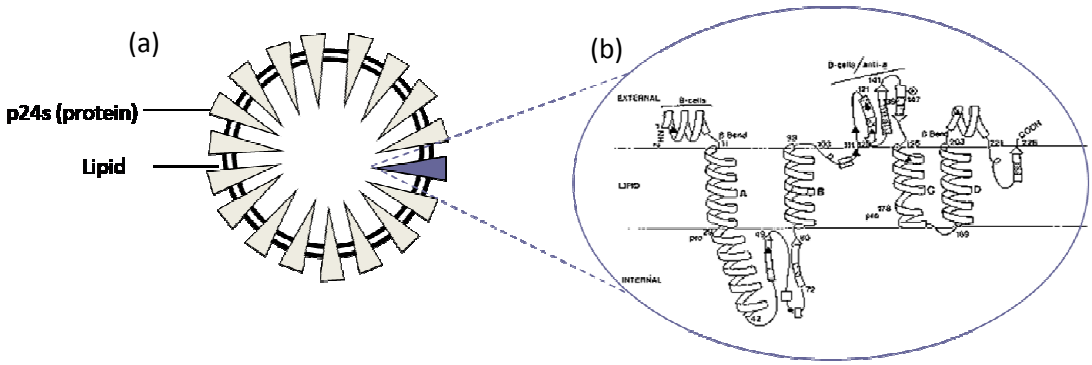


***Figure 1.2**: **(a)** shows the cross-section of an HBsAg particle adapted from Koistinen (1980) while **(b)** illustrates the interaction between a p24s protein monomer and the lipid bilayer on the surface of the HBsAg particle (image taken from Mahoney and Kane, 1999).*

The recombinant HBsAg particles are cysteine-rich and the structural evolvement and maturation depends on the level of disulphide formation. The formation of correct intra- and intermolecular disulphide bonds are critical as they determine the conformational stability and immunogenicity of HBsAg vaccines (Guerrero *et al.*, 1988; Zhao *et al.*, 2006). HBsAg derived from human blood plasma are already in the mature form but in recombinantly expressed HBsAg, the disulphide bond formation and the cross-linking process continues during purification. Additional steps of treatment with potassium thiocyanate (KSCN) and storage at elevated temperatures have been included at the end of the purification chain to complete the maturation process of recombinant HBsAg (Zhao *et al.*, 2006).

HBsAg characterisation studies have revealed that the particle is composed of ~ 75% protein and ~ 25% lipid by mass (Dreesman *et al.*, 1972; Gavilanes *et al.*, 1982). The lipid-protein interactions are responsible for the formation of the desirable helical structures of the proteins forming the epitopes (Gavilanes *et al.*, 1990) and this is shown

schematically in **Figure 1.2(b)**. Sterol accounts for 30% of the lipid constituent of the HBsAg particle while phospholipids account for the remaining 70%, of which phosphatidylcholine (PC) is the most abundant. Negatively charged phospholipids, phosphatidylserine (PS) and phosphatidylinositol (PI), in particular, have been shown to have significant influence on HBsAg activity (Gomez-Gutierrez *et al.*, 1994). A full account of the role of lipids in maintaining the structure and antigenicity of HBsAg and the effects of lipid reconstitution is available elsewhere (Gavilanes *et al.*, 1990; Gomez-Gutierrez *et al.*, 1994).

The intrinsic characteristics of the HBsAg particles, outlined above, are critical considerations in ensuring product quality and potency. Like other VLPs, HBsAg particles are large macromolecular entities which are difficult to characterise and are susceptible to conformational and biological changes in different microenvironments. As previously discussed, the process of producing the vaccine itself often defines the end product (Buckland, 2005) and regulatory demands are stringent considering that the vaccine products are administered to healthy individuals. It is required that assurance of the identity and composition of the administered dosage is provided to meet the demands of product safety and efficacy. Hence, in this study, the biological and physiochemical aspects of the HBsAg system are carefully considered alongside the purification process development efforts. Ultra-scale down (USD) techniques are particularly valuable here in simulating industrial scale conditions to allow the characterisation of the biochemical and biophysical responses of the HBsAg particles to different processing environments.

1.3.5 Hepatitis B Surface Antigen (HBsAg) platform for novel vaccines

In addition of their use as direct immunogens, VLPs have shown enormous potential as platforms for the development of future generation vaccines based on hybrid or chimeric VLP. VLPs are highly efficient in stimulating cellular and humoral responses as they provide the spatial structure for the display of conformational epitopes that mimic the native viral structure leading to enhanced antibody production (Grgacic and Anderson, 2006).

The hepatitis B surface antigen (HBsAg), with the ability to self-assemble with host-derived lipids into empty non-infectious VLPs (Cheong *et al.*, 2009), is attractive as a delivery platform for foreign epitopes. The efficiency of the HBsAg particles in priming cellular and humoral responses even in the absence of an adjuvant has been reported (Boisgerault *et al.*, 2002). Chimeric vaccines for Hepatitis C (Netter *et al.*, 2001), dengue (Bisht *et al.*, 2001) and HIV (Michel *et al.*, 2007; Greco *et al.*, 2007) have been developed using this platform.

The HBsAg particles are composed of a lipid bilayer surrounded by external hydrophobic loops which could be genetically engineered to carry the heterologous antigens for delivery (Delpeyroux *et al.*, 1986; Netter *et al.*, 2001; Phogat *et al.*, 2008). The ability of the hydrophobic loops to accept peptides facilitates the insertion of selective epitopes against diseases including HIV-1 and hepatitis C (Lee *et al.*, 1996; Schlienger *et al.*, 1992; Boisgerault *et al.*, 2002).

The lipid-envelope structure of the HBsAg is an attractive attribute as it allows the incorporation of foreign antigens requiring the support from a lipid bilayer structure. This aspect is particularly relevant in the development of HIV vaccines where a lipid membrane environment is necessary to orientate the antigenic epitopes for efficient

induction of broadly neutralising antibodies (Grunder *et al.*, 2002). Preliminary studies showed that the HIV epitopes could be appended to the C-terminus of the HBsAg S-1 protein and the lipid environment provided the support necessary for effective antibody binding (Phogat *et al.*, 2007). Similarly, the chimeric VLP for dengue virus envelope protein showed enhanced immunogenicity when assembled on the HBsAg platform (Bisht *et al.*, 2002).

HBsAg particles have a hollow core with an encapsulation space of 900-8000 nm^3 (Reimann *et al.*, 2006) and access to the interior is mediated by pores in the bilayer. The hollow structure of the HBsAg particles could also be employed for the encapsulation or entrapment of antigenic proteins and peptides, oligonucleotides or cytokines which would be exposed to the surface by virtue of the pores. Early proof-of-concept studies for this HBsAg application have been demonstrated (Reimann *et al.*, 2006).

1.4. Overview of production and purification methods for HBsAg

1.4.1 HBsAg expression in *S. cerevisiae*

The HBsAg particles used as the VLP model in this study was developed by Merck (West Point, PA, USA) using *S. cerevisiae* as the host cell expression system. Details of the plasmid and strain construction are reported elsewhere (Carty *et al.*, 1989; Hinnen *et al.*, 1978).

The crucial starting point in the development of a VLP vaccine is the design of a cell based system which is stable over many generations and produces high titres of the

VLP product (Buckland, 2005). The choice of the expression system is particularly important for lipid-envelope VLPs for the reason that the lipid constituents are derived from the host cell (Betenbaugh *et al.*, 1995; Buonaguro *et al.*, 2005). Although the production of HBsAg has also been achieved using bacteria (Shu *et al.*, 2006), transgenic plants (Kumar *et al.*, 2005) and mammalian cells (Diminsky *et al.*, 1997), *S. cerevisiae* remains a highly attractive host. Yeast fermentation is well established, highly reproducible and amenable to large scale fermentation (Buckland, 2005). Additionally, yeast cells have the ability of performing complex eukaryotic-like post-translational modifications producing proteins similar to those of the mammalian origin and a wealth of genetic information is available for this strain which facilitates the engineering of the desired cell metabolism and expression characteristics for the VLP.

Fermentation conditions for VLP production would depend on the host expression system chosen. The recombinant HBsAg used as the VLP model in this study is transcribed under the control of the GAL-10 promoter in *S. cerevisiae*. Hence, HBsAg expression is regulated by the levels of glucose and galactose in the culture media. Fermentation conditions and glucose-galactose interactions in HBsAg expression have been reported previously (Carty *et al.*, 1987; Carty *et al.*, 1989). For plasmid selection and maintenance, the Merck HBsAg strain also carries a Leu^+ gene in the same plasmid as the HBsAg gene. This allows the use of a leucine-free basal media for fermentation which would select for cells containing the plasmid with the Leu^+ and HBsAg genes. Fermentation for HBsAg production is typically carried out in a batch process (Carty *et al.*, 1987) but the use of continuous fermentation has also been reported (Fu *et al.*, 1995).

An issue for consideration when developing yeast fermentation media is that yeast cells are susceptible to catabolite repression, a condition where cell growth is

hindered when excess sugars such as glucose are present (Gancedo, 1998). Oura (1974) developed a fully defined media for yeast fermentation using glucose that has minimal catabolite repression effects. Yau (2005), in her MEng research project in Biochemical Engineering (UCL), proposed a media for HBsAg production which has the combined advantages of the Oura (1974) and the Fu *et al.* (1995) media. This media was shown to be successful for the fermentation of wild type *S. cerevisiae* cells but was not tested for the recombinant HBsAg strain due to time constraints. Joyce *et al.* (1998) reported on a recombinant *S. cerevisiae* fermentation media which is semi-defined for the production of the human pappilomavirus (HPV) vaccine. It is known that the *S. cerevisiae* host expression system for this vaccine is similar to that for hepatitis B and hence the media may be employed for HBsAg production.

In this research, the recombinant *S. cerevisiae* for HBsAg was donated by Merck (West Point, PA, USA) in the form of a master seed stock. Fermentation is not a key element in this project however it is crucial to produce sufficient feed material for subsequent purification studies which were representative of material in an industrial process. There is limited information in the public domain on fermentation media for HBsAg production using the Merck recombinant *S. cerevisiae* strain. To minimise time and research effort in generating feed stock for purification studies, prior knowledge from the Yau (2005) and Joyce *et al.* (1998) studies were used as the basis for fermentation development.

1.4.2 Recovery and purification of HBsAg from *S. cerevisiae*

HBsAg is expressed in *S. cerevisiae* as an intracellular product and the typical recovery and purification process is summarised in **Figure 1.3**. For recovery of the

intracellular HBsAg product, the yeast cells following fermentation are harvested, concentrated and washed with buffer for the removal of media components and antifoam. This is usually accomplished using centrifugation or microfiltration and the cells are stored as cell paste at -70 ^{o}C (Sitrin and Kubek, 1992).

Product recovery is performed by suspending a batch of frozen cells in a buffer containing phenylmethylsulfonyl fluoride which is a protease inhibitor (Dekleva, 1999), and the suspension is subjected to cell disruption by homogenisation. Following cell breakage, the crude extract is treated with a phosphate buffer containing a detergent, usually Triton X-100 (Wampler *et al.*, 1985). This is a critical step in the recovery of HBsAg as the detergent facilitates the liberation of the HBsAg from tightly associated endoplasmic reticulum (ER) membrane components (Dekleva, 1999). Removal of cell debris can be performed by centrifugation (Wampler *et al.*, 1985) or microfiltration (Deklava, 1999). Residual detergent is removed by recirculation through polystyrene XAD-4 beads and the product stream can be ultrafiltered through a 100-kDa membrane to clear small molecular weight contaminants and for product concentration.

Purification is typically accomplished by chromatography-based operations (Dekleva, 1999; Wampler *et al.*, 1985; Belew *et al.*, 1999) although the use of precipitation and density ultracentrifugation has been reported (Deml *et al.*, 1999). The HBsAg product is initially purified via adsorption and elution from colloidal silica (Aerosil) (Wampler *et al.*, 1985). Conditions for this process are reported elsewhere (Pillot *et al.*, 1976; Sitrin and Kubek, 1992). Final polishing is performed using hydrophobic interaction chromatography using butyl agarose (Dekleva, 1999).

As highlighted by Zhao *et al.* (2006), the HBsAg produced in *S. cerevisiae* is not in the fully disulphide-bonded form as found in blood-derived HBsAg. The maturation

process for recombinant HBsAg progresses through the purification process. To facilitate the formation of disulphide cross-linkages in the mature form, the HBsAg particles are treated with thiocyanate and incubated at an elevated temperature of 37 ^{o}C prior to product formulation (Zhao *et al.*, 2006). The product is finally adjuvanted by co-precipitation with aluminium hydroxide (Dekleva, 1999).

In the complex multistage operations involved in HBsAg production and purification, product yield is dictated by the efficiency of the detergent-mediated HBsAg liberation step while the purification performance of membrane filtration and chromatographic operations are influenced by the level of contaminants such as yeast proteins and lipids. This thesis investigates the development of an improved primary purification strategy to achieve higher HBsAg recovery and a cleaner output to reduce the burden on downstream operations.

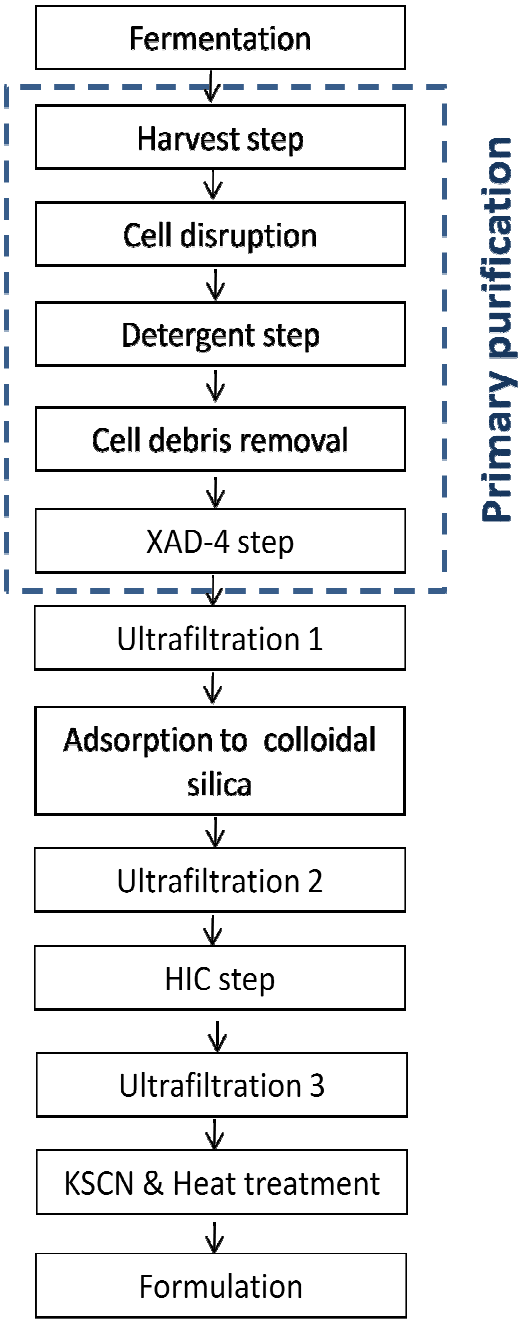


***Figure 1.3**: Typical process flowsheet for HBsAg production and purification adapted from Dekleva (1999). The primary purification stage which is of specific interest in this study is highlighted (- - - -).*

1.5 Areas of focus in improving primary recovery and purification

1.5.1 Detergent-mediated HBsAg liberation

The HBsAg product remains permanently localised on the yeast endoplasmic reticulum (ER) following protein translation as transport through the secretion pathway is blocked (Biemans *et al.*, 1992). A detergent is required to facilitate the liberation of HBsAg from tightly associated ER membrane components (Dekleva, 1999). Typically Triton X-100, a non-ionic detergent, is employed for this purpose although alternative detergents such as polysorbate 20 and 80 (PS 20 and PS 80) and variants of Triton such as Triton X-101, CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) and glycol ether solvents have been reported for similar applications (Wijnendaele *et al.*, 1987; Kniskern and Hagopian, 1997; Allen *et al.*, 2007).

The key feature to a detergent's function is in its amphipathic structure comprising a hydrophilic "head" region and a hydrophobic "tail" region which can associate with the hydrophobic surfaces of proteins. Detergents facilitate the recovery of membrane proteins by disrupting the bipolar lipid membrane of cells and by forming protein-detergent complexes which are soluble. Of the detergents commonly employed for the recovery of lipid-envelope VLPs, Triton and polysorbate, both fall under the non-inonic detergent class. Their difference lies in the polymer type and length of the hydrophobic "tails". Triton detergents are made from ethyleneglycoether polymers and have shorter hydrocarbon chains and higher CMC values than polysorbate detergents which contain oxyethylene polymers (Caligur, 2008). Detergents with lower CMC values are more stable however the interaction between the detergent and the protein is also stronger which could be an issue for subsequent detergent removal efforts. CHAPS in contrast is a zwitterionic detergent which may have the added benefit of being more

effective for disrupting protein-protein bonds in reducing aggregation due to its ionic nature although having a net neutral charge. In general, anionic or cationic detergents are less preferred for biological applications as their charge characteristics could modify protein structure leading to increased risks of protein denaturation. Specifically for HBsAg liberation, there are several hypotheses as to how the detergent functions, mechanistically. Chi et al (1994) proposed that detergents weaken the hydrophobic interactions that exist between the VLP and the membrane components. According to Sitrin and Kubek (1992), detergents facilitate the disassociation of the HBsAg from the ER by promoting the separation of the yeast ER membrane from other unwanted cellular debris. A study by Smith and co-workers suggests that detergent may also promote VLP vesicle formation by inward budding of the ER membrane (Smith *et al.*, 2002; Kreibich *et al.*, 1982).

From the above, it appears that high detergent concentrations would favour HBsAg liberation based on reaction kinetics. However, to be taken also into consideration, is the physiochemical nature of the HBsAg particles which are 75% protein and 25% lipid by mass (Gavilanes *et al.*, 1982). There has been a lack of consensus as to the optimal concentration of detergent for this purpose. Other groups have reported that excess detergent could lead to HBsAg particle delipidation (Skelly *et al.*, 1981; Howard *et al.*, 1982; Sanchez *et al.*, 1983) which in turn could result in a loss of antigenic activity. This is unsurprising given that the lipid moiety has significant roles in the maintenance of the structural and antigenic properties of the protein components of the HBsAg (Gavilanes *et al.*, 1990).

The amount of detergent could also have indirect implications on the contamination profile of the resulting process stream. Large amounts of lipid are produced by yeast cells over extended periods of cell culture (Wijnendaele *et al.*, 1987)

which in the case of HBsAg production is necessary to achieve the desired product titres. The majority of these lipids are associated with membranous organelles which also contain some proportion of proteins for specific metabolic functions. During the detergent step, it is likely that the membrane associated lipids and proteins are co-released alongside the HBsAg product and the extent of co-liberation is also a function of detergent concentration.

Based on the factors highlighted above, it is critical to identify a suitable detergent and its optimal range of operating concentrations for effective recovery of HBsAg, preservation of its antigenic properties and minimal co-release of contaminating host lipids and proteins.

1.5.2 Precipitation for partial purification

Precipitation is a traditional protein purification method and is examined here as a means of achieving partial purification and enrichment of HBsAg prior to higher resolution chromatographic operations. Polyethylene glycol (PEG) and ammonium sulphate are the most widely cited precipitating agents for VLPs and are assessed for the primary purification of HBsAg based on the degree of contamination reduction and product yield.

Polyethylene glycol (PEG) is attractive as a precipitating agent owing to its low potential for protein denaturation even when present at high concentrations (Ingham *et al.*, 1990; Tsoka *et al.*, 2000) and its high fractionation efficiency when purifying large size proteins such as VLPs (Juckes *et al.*, 1971). The application of PEG precipitation for HBsAg purification is not entirely new. Purification of the first generation hepatitis B vaccine based on HBsAg from plasma of carriers relied on multiple PEG precipitation

"cuts". Since the recombinant HBsAg examined here is not identical to the human form due to differences in disulphide cross-linking, these recombinant particles may have different solubility behaviours in the presence of PEG. There is also a difference in the role of the PEG precipitation step. In the 1970s process for human HBsAg, PEG precipitation was the purification workhorse and multiple "cuts" were necessary to achieve the desired final purity, often at the expense of significant product loss. As a primary purification process for future-generation vaccines, its role would be to reduce the contamination burden and process volume entering downstream operations in a single "cut" with minimal product loss. For PEG precipitation as a primary purification step product yield is critical and this relies on the effectiveness of precipitate recovery from the liquor. Although PEG has a low intrinsic viscosity (Polson *et al.*, 1964), concentrations above 10% w/v PEG may have substantial effect on the viscosity of the process stream. The effect of this on the performance of the centrifugation or filtration step for the precipitate recovery would need to be evaluated.

The use of ammonium sulphate, another popular precipitating agent, is also investigated. It is generally known that the ammonium sulphate salt is less selective compared to PEG which results in larger quantities (> 40% w/v) of the salt being required for protein precipitation (Wijnendaele *et al.*, 1987) which could create waste disposal issues. Lipid solubility, in contrast, is more sensitive to ammonium sulphate and lower concentrations of the salt are typically sufficient for their precipitation. Bracewell *et al.* (2008) demonstrated an inverse purification strategy using ammonium sulphate precipitation. In the study, lipid contaminants were precipitated out of solution whilst protein products are retained in the soluble phase. This strategy would be more suitable for large-scale VLP purification owing to the lower levels of ammonium sulphate consumption which would pose less waste disposal issues.

1.5.3 Selective product release for primary purification

For expression of recombinant proteins in *S. cerevisiae*, there are three main regions in the cell where the product of interest is likely to be located: (1) the space between the cytoplasmic membrane and the outermost mannan-protein layer of the cell wall, (2) in the cytosol, or (3) embedded in subcellular organelles or particles (Huang *et al.*, 1991). It is within the third category that the production of recombinant lipid-envelope VLPs such as the HBsAg falls. The natural localisation of the product of interest in a specific region of the cell can provide a powerful means of recovering the product separately from bulk contaminants originating from the host cell. This approach has been demonstrated previously in the recovery of proteins from the periplasmic space of *E.coli* cells using osmotic shock or enzymes (French *et al.*, 1996; Witholt *et al.*, 1976) and from specific regions in yeast using detergent and enzymes (Chi *et al.*, 1994; Huang *et al.*, 1991; Asenjo *et al.*, 1993). Using these methods, several-fold product enrichment was reported with product yields of up to 90%.

As highlighted previously, HBsAg particles in yeast are permanently localised on the endoplasmic reticulum (ER) because transport through the secretion pathway is blocked (Biemans *et al.*, 1992). It is for this reason that a detergent is typically added to the cell lysate following homogenisation to release the HBsAg from the associated ER components. The ability to separate ER particles containing the HBsAg product from bulk cell contaminants prior to the addition of detergent would be invaluable as this would allow the HBsAg product to be recovered later and in a cleaner process stream. The endoplasmic reticulum which is slightly hydrophobic (Chi *et al.*, 1994) and denser than the cell cytosol would be sedimented together with solid debris particles when centrifuged whilst bulk cytosolic material would be in the supernatant. By discarding the supernatant fraction and treating only the solids fraction with detergent, HBsAg

would be released into a process stream with significantly reduced host lipid and protein contaminants. The efficiency of this process would rely on the relative partitioning of the HBsAg product and host contaminants across the cytosol and the membranous components.

It is possible to enhance further the selective recovery process by fine-tuning the cell disruption conditions. Characterisation of the size distribution of yeast debris generated as a function of the homogenisation conditions revealed that the release of intracellular proteins is often accompanied by fragmentation and micronisation of the cell wall (Siddiqi *et al.*, 1995). Conditions of the homogenisation step could have a major influence on the efficiency of the selective recovery process at three different levels: (1) the extent of cell disruption would influence the accessibility of detergent to the ER during the HBsAg liberation step and this would impact product yield, (2) the population size of the ER components following homogenisation would influence the effectiveness of their recovery by sedimentation during the initial centrifugation step and this would also impact product yield, and (3) the particle size distribution of membranous debris would influence the rate of co-extraction of contaminating lipids and proteins during the detergent step and this would impact the level of purification achieved. Thus, a trade-off study on the impact of homogenisation conditions would be warranted in developing any approach to selective product release.

1.5.4 Scalability of selective product release

In process development, USD techniques are invaluable as they are capable of predicting industrial scale performance of biopharmaceutical protein downstream processing in a rapid fashion using as little as millilitres of material (Titchener-Hooker

et al., 2008). Identification of process challenges and prediction of performance of large scale protein precipitation operations using ultra scale-down techniques has been demonstrated (Boychyn *et al.*, 2000). Hence, the focus here was on the scale-up of a selective product release method.

The selective product release methodology investigated in this work relies on a twin centrifugation operation. The role of the first centrifugation step is to allow the elimination of bulk cytosolic contaminants whilst retaining the solids fraction containing the HBsAg product. The second centrifugation step which is performed following detergent treatment recovers the HBsAg product in the supernatant whilst solid contaminants are discarded. Different centrifuge designs such as the tubular bowl, multichamber bowl and disc-stack centrifuge have been reviewed (Salte *et al.*, 2006; Boychyn *et al.*, 2004). The CARR Powerfuge^{TM} centrifuge, which is a tubular bowl variant, is most suited for inclusion in the selective product release process sequence due to its good solids compaction and high clarification features. The level of dewatering and clarification attained would dictate the selectivity and product yield from this approach.

The Sigma concept (Ambler, 1959) allows the comparison and prediction of centrifuge performance across scales and centrifuge designs by the introduction of correction factors which account for differences in flow patterns. USD models have been developed from the Sigma theory (Tustian *et al.*, 2008; Boychyn *et al.*, 2004; Boychyn *et al.*, 2000; Salte *et al.*, 2006) and are useful here to predict the clarification performance of an industrial CARR Powerfuge^{TM} operating at different feed flow rates and rotational speeds. Having determined the optimal operating conditions of the CARR Powerfuge^{TM} centrifuge for the selective recovery process, a pilot-scale validation study

can be performed to verify that the purification potentials of any methodology are not compromised upon scale-up.

1.6 Research Aims

The fundamental goal of this project is to investigate a series of potential improvements for primary purification processes for future generation lipid-envelope VLP vaccines from *Saccharomyces cerevisiae* with the purpose of reducing the burden on higher resolution operations downstream and to achieve better overall process performance. The critical response factors applied here when measuring the efficiency of primary purification are: (1) product yield from primary purification; and (2) effective reduction of host-derived contaminants, specifically yeast lipids and proteins. These studies will be carried out using the hepatitis B surface antigen (HBsAg) as the lipid-envelope model to achieve the following objectives:

1.6.1 Identification of suitable detergent and range of operating conditions

The importance of the detergent-mediated VLP liberation step is unparalleled in setting the benchmark for VLP yield and antigenicity and the contamination profile of the process stream entering subsequent purification. To identify the detergent most suited for this purpose, the effectiveness of a range of commonly employed detergents will be compared. Following this, a trade-off study will be performed to identify a suitable operating range for the detergent step. Factors including the effects of detergent concentrations on HBsAg yield, antigenic activity, physical characteristics of the

HBsAg, the level of release of host cell contaminants (lipids and proteins) and the potential impact on the performance of a downstream ultrafiltration operation will be taken into account in this evaluation.

1.6.2 Investigating the potentials of PEG and ammonium sulphate precipitation for primary purification

PEGs with molecular weights of 6000 and 8000 have been reported (Tsoka *et al.*, 2000) to be most effective for VLP precipitation. To establish the suitable precipitating conditions, different concentrations of PEG 6000 and PEG 8000 will be compared in terms of their effectiveness in promoting HBsAg precipitation and the resulting degree of product enrichment. The use of centrifugation and filtration will be compared to determine their suitability for precipitate recovery. Finally, the practical and economical feasibility of PEG precipitation as a primary purification will be assessed based on the overall step yield and the purification factor achieved.

Ammonium sulphate is known to be less selective than PEG and higher concentrations (> 40% w/v) are typically required for protein precipitation (Wijnendaele *et al.*, 1987). Its application at lower concentrations however could facilitate lipid aggregation and removal (Bracewell *et al.*, 2008). An inverse strategy is investigated here in which low concentrations are employed to precipitate and remove lipid contaminants while maintaining the HBsAg product in solution. Hence, screening studies would be performed to identify ammonium sulphate concentrations which achieved highest lipid precipitation with maximum HBsAg solubility. The degree of HBsAg enrichment and product yield will be evaluated to determine the practical and economical feasibility of this process.

1.6.3 Investigating the potential of selective product release for primary purification

To establish the effectiveness of any improved method an analysis of the relative partitioning of the HBsAg product and yeast-derived contaminants between the supernatant and solids fraction immediately following cell disruption and centrifugation would be required. As previously discussed, homogenisation pressure conditions could impact the effectiveness of contaminant removal. Hence a subsequent study could be performed to identify disruption pressure conditions which afford the best trade-off between product yield and effective reduction of contaminants. To provide a quantitative demonstration of the value of this process, a HIC chromatography challenge could be performed to analyse the effects of a cleaner feed stream on product binding and chromatography yields.

1.6.4 Scale-up study of selective product release process

A tubular bowl CARR Powerfuge P6^{TM} shall be employed for the pilot-scale demonstration of the selective product release methodology which is based on a twin centrifugation strategy. To predict centrifugation conditions for a pilot-scale process, ultra-scale down (USD) methods developed previously (Tustian *et al.*, 2008; Boychyn *et al.*, 2004; Boychyn *et al.*, 2000; Salte *et al.*, 2006) will be adopted. Using optimal conditions predicted using the USD methods, the process will be demonstrated at pilot-scale to evaluate its efficiency upon scale-up. Comparison of USD and pilot-scale results will also provide an assessment on the reliability of USD predictions.

1.7 Organisation of thesis

The first chapter provides an introduction to the project and discusses the current development and challenges facing VLP production and purification. The relevance of studying the purification of the hepatitis B surface antigen (HBsAg) as a VLP model and how this project could benefit the development of future generation lipid-envelope VLPs were described here. A literature review on the history, biology and downstream processing methods for VLPs in general and specifically the HBsAg particle were also presented.

The second chapter provides a compilation of the materials and methods employed in this study. This chapter will serve as the reference point for discussions of experimental and analytical procedures in all of the subsequent results chapters.

The third chapter describes how fermentation development studies were conducted to design a suitable protocol for pilot-scale production of recombinant *S. cerevisiae* cell stock for purification studies. Comparison studies on two different media compositions previously developed (Yau, 2005 and Joyce *et al.*, 1998) as well as fermentation scale-up from 20 L to 75 L are described here.

The fourth chapter reports on fundamental studies performed to characterise and improve the performance of detergent-mediated HBsAg liberation from the endoplasmic reticulum (ER). Screening studies comparing the effectiveness of different detergents followed by a trade-off analysis to determine the optimal detergent concentration range are presented here. Key discussion points include the effect of detergent on HBsAg yield, antigenic activity, level of co-extraction of contaminating host proteins and lipids and the potential impact on the performance of a downstream ultrafiltration operation.

The fifth chapter investigates the potentials of polyethylene glycol (PEG) or ammonium sulphate precipitation as a primary purification step to reduce the level of lipid and protein contaminants entering downstream operations. Different concentrations of PEG 6000 and PEG 8000 and ammonium sulphate were analysed based on their purification potentials and step yields. For PEG precipitation, centrifugation and filtration were compared as separation options for precipitate recovery.

The sixth chapter explores the potentials of primary purification using a selective product release approach which is based on a twin centrifugation strategy. The relative partitioning of the HBsAg product and host lipid and protein contaminants across the supernatant and solids fractions immediately following cell disruption and centrifugation are characterised. The impact of varying homogenisation pressure conditions to drive the selectivity of the process higher is also demonstrated here. The purification potential of this methodology was assessed based on HBsAg product enrichment and yield as well as the benefits to a downstream chromatographic step.

The seventh chapter follows from the successful demonstration of the selective recovery methodology and is aimed at evaluating its amenability to scale-up. The application of USD techniques for predicting suitable operating conditions for key centrifugation operations for a pilot-scale process are detailed here. Discussion also includes a comparison of the performance at pilot-scale against USD predictions and an overall evaluation of the selective product recovery methodology upon scale-up.

The eighth chapter summarises the findings from this study and concludes on the contributions of this thesis. Areas of focus for future work are also outlined here.

The final chapter contains the inventory of references accessed in this thesis.

Chapter 2

Materials and Methods

Materials

All chemicals employed were purchased from Sigma-Aldrich (Dorset, UK) and were of analytical grade unless stated otherwise.

2.1 Fermentation development studies

2.1.1 *S. cerevisiae* cell lines

Wild type *S. cerevisiae* ATCC 26786 (ATCC, Middlesex, UK) was employed for a trial fermentation run. Subsequent studies were performed using a recombinant *S. cerevisiae* strain (Merck & Co. Inc, West Point, PA, USA) which produced the HBsAg product.

2.1.2 Fermentation media evaluation

Two previously reported fermentation media were investigated here for the production of HBsAg from recombinant *S. cerevisiae*. Media composition and preparation methods are detailed in the following sections.

2.1.2.1 Media developed by Yau (2005)

Shake flask seed cultures stages were prepared using a complex media containing 20 - 40 g/L glucose, 10 g/L glycerol, 10 g/L yeast extract, 0.4 g/L adenine and 0.2 g/L uracil. The HBsAg production stage was carried out using defined media adapted from Oura (1976) and Fu *et al.* (1996) containing 20 g/L glucose, 10 g/L succinic acid, 30 g/L monosodium glutamate, 0.4 g/L adenine and 0.2 g/L uracil as the main components. These components, other than glucose which was sterilised in an autoclave, were sterilised in-situ in the fermenter. Vitamins containing 0.125 mg/L biotin, 5 mg/L thiamine, 6.25 mg/L panthothenic acid, 125 mg/L meso-inositol, 6.25 mg/L pyridoxine and 5 mg/L nicotinic acid and trace elements of 120 mg/L potassium chloride, 60 mg/L sodium chloride dehydrate, 90 mg/L calcium chloride pentahydrate, 3.8 mg/L manganese sulphate monohydrate, 0.003 mg/L sodium molybdate dehydrate, 0.002 mg/L cobalt sulphate heptahydrate, 0.002 mg/L cobalt sulphate heptahydrate, 0.002 mg/L zinc sulphate heptahydrate, 0.007 mg/L boric acid, 0.002 mg/L potassium iodide and 0.003 mg/L nickel sulphate hexaphydrate were filter sterilised into the fermenter. An iron – phosphate source containing 31 mg/L iron (II) sulphate heptahydrate dissolved in diluted phosphoric acid was also added. At the HBsAg production stage, 20 g/L galactose, prepared and autoclaved separately, was added for induction.

2.1.2.2 Media developed by Joyce *et al.* (1995)

This is a semi-defined media originally for the production of the HPV vaccine in *S. cerevisiae*. Media compositions were identical for both the seed and the HBsAg production stages, containing 20 - 40 g/L glucose as the main carbon source, 8.5 g/L Difco yeast nitrogen base without amino acids and ammonium sulfate; 0.2 g/L adenine;

0.2 g/L uracil; 10 g/L succinic acid; 5 g/L ammonium sulfate; 40 g/L glucose; 0.25 g/L L-tyrosine; 0.1 g/L L-arginine; 0.3 g/L L-isoleucine; 0.05 g/L L-methionine; 0.2 g/L L-tryptophan; 0.05 g/L L-histidine; 0.2 g/L L-lysine; 0.3 g/L L-phenulalanine. For the VLP production stage, 40 g/L galactose was added at the start of fermentation to induce HBsAg expression.

2.1.3 Cell expansion and cell banking methods

Cell expansion for wild type *S. cerevisiae* ATCC 26786 (ATCC, Middlesex, UK) was carried out by adding 1 mL of the master cell bank into 50 mL of media containing 10 g/L succinic acid, 20 g/L galactose, 30 g/L monosodium glutamate and 10.4 g/L glycerol in a 250 mL shake flask. This was left overnight in a shaker incubator at 28 ^{o}C with agitation of 250 rpm to achieve OD > 2 Au. 50% glycerol was added to the media in a 1:2 v/v ratio and the culture was aliquoted in 1 mL volumes for storage at -80 ^{o}C. For the recombinant *S. cerevisiae* producing HBsAg (Merck & Co. Inc, PA, USA), cell expansion was carried likewise but in the media described by Joyce *et al.* (1995) and without glycerol addition prior to storage.

2.1.4 Development of fermentation protocol for HBsAg production

The seed stage involved growing a frozen vial (1 mL) of the cell bank in 50 mL of media in a 250 mL shake flask for 24 h in a shaker incubator at 28 ^{o}C with agitation of 250 rpm. 4 mL of this culture was subsequently transferred to 396 mL of media in 2 L shake flasks and grown for a further 24 h under the same conditions. [To seed a 20 L fermenter, 3 of these 2 L shake flasks would be required while at the 75 L scale, 12 would be required.]

Fermentation studies were performed at 20 L scale using the LH 2000 Insitu Inceltech fermenter (Inceltech UK Ltd, Slough, UK) which has the following dimensions: vessel height = 510 mm, vessel diameter = 230 mm, shaft height = 490 mm, shaft diameter = 12.7 mm, number of impellers = 3, impeller diameter = 68.7 mm, and baffle dimensions = 355 x 22 x 1.8 mm. The successful fermentation was scaled-up to 75 L using the Inceltech High Contamination fermenter (Inceltech UK Ltd., Slough, UK) which has the following dimensions: vessel height = 925 mm, vessel diameter = 327 mm, shaft height = 595 mm, shaft diameter = 26 mm, number of impellers = 3, diameter of impeller = 105.5 mm, and baffle dimensions = 612 x 32 x 3.5 mm. Fermentation working volumes were set at 60-80% and inoculation was performed with 10% working volume of the fermenter. For timed-based induction, pre-autoclaved galactose of the same initial concentration as glucose was added for induction when the glucose level had depleted. For auto-induction, galactose was added together with glucose at the start of fermentation. Fermentation was carried out for a total of 48 to 90 h with pH maintained at 5.5 ± 0.1 with automated additions of 2 M H_2SO4 and 4 M NaOH. Fermentation level was monitored using a foam probe with automated additions of antifoam. Measurements of temperature, pH, agitation speed, dissolved oxygen tension (DOT) and airflow rate were recorded automatically using the ProPack data logging and acquisition software (Acquisition Systems, Hampshire, UK) integrated to the fermenter. Regular samplings were performed to allow off line analysis of optical density (OD) measurements at 600nm, dry cell weight analysis and glucose analysis.

2.2 Pilot-scale production of HBsAg feed stock for purification studies

Following from the fermentation studies, a protocol was established for the production of HBsAg feed stock for subsequent purification investigations. This protocol is based on growing the *S. cerevisiae* strain for HBsAg (Merck & Co. Inc, West Point, PA, USA) using the Joyce *et al.* (1995) method described in **Section 2.1.2**. To generate sufficient material for purification experiments, multiple fermentation batches were carried out at the 75 L scale using the protocol described in **Section 2.1.4**.

Following fermentation, cells were harvested using a CARR Powerfuge^{TM} P6 (Pneumatic Scale Corporation, Florida, USA) tubular bowl centrifuge which has a 1 L bowl volume and is operated in a batch mode. For cell harvesting, the centrifuge rotational speed was set at 15,000 rpm and feed flowrate at 1 L/min. Cells recovered in the form of a solid paste were stored in polyethylene bags at -80 ^{o}C.

2.3 Pilot-scale homogenisation for cell disruption

Homogenisation was performed using a Gaulin Micron Lab 40 homogeniser (APV Gaulin GmbH, Lubeck, Germany), which has a 40 mL capacity, for all studies unless specified. Prior to homogenisation, the cell paste was resuspended in 0.1 M sodium phosphate, pH 7.2, with 0.5 M NaCl containing phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, prepared in to a concentration of 0.2M in isopropanol and added to the phosphate buffer to achieve a final concentration of 2 mM of PMSF. Cell paste to buffer ratio for resuspension was 1:3 (w/v). The temperature of the machine

was maintained at below 6 ^{o}C by flowing glycol coolant through the external coils throughout the process. Homogenisation was performed for 3-4 passes at 1200 bar unless stated otherwise.

2.4 Detergent treatment for HBsAg liberation

In experiments for comparing the efficiency of different detergents and chemicals for HBsAg liberation, solutions of Triton X-100, Triton X-101, Polysorbate 20 and 80 (PS20 and PS80) and CHAPS were prepared in their typical operating concentrations of 0 – 1% v/v and ethyl butyl ether (EB) in the range of 0 – 30% w/v in 0.01 M phosphate buffer (pH 7.5). Yeast cell homogenate was added to 2 volumes of the solutions prepared and these were allowed to mix for 4 h on an Eppendorf Thermomixer (Eppendorf, Cambridge, UK). The removal of cellular material was performed subsequently by centrifugation (Eppendorf Centrifuge 5415R, Cambridge, UK) at 13,000 rpm for 15 min. The supernatant samples were recovered for analysis.

For all other studies, Triton X-100 detergent, which was found to give the highest degree of HBsAg liberation, was employed. For screening studies to determine the optimal working concentration of Triton X-100, solutions of Triton X-100 in 0.01 M phosphate buffer (pH 7.5) were prepared in the range of 0 – 10% v/v. The Triton X-100 solutions were added to the yeast cell homogenate in a 2:1 volume ratio to achieve resulting Triton X-100 concentration of 0 – 6.7 % v/v. Incubation with Triton X-100 and centrifugation for the removal of cell debris were carried out as described above. Prior to analysis, to prevent interference of Triton X-100 with the assays, excess detergent was removed by incubating the samples with Amberlite XAD-4 beads in a

batch mode (Supelco, Dorset, UK) for 2 h. [The amounts of XAD-4 required were based on 0.5 g XAD-4 per g Triton X-100 determined from the Sigma product information sheet. The efficiency of Triton X-100 removal was verified using control experiments.] The XAD-4 beads are larger than the tip of a 200 µL pipette tip, hence following the XAD-4 step, samples were recovered by careful pipetting avoiding the XAD-4 beads.

To generate larger amounts of HBsAg feed material required for purification studies, the detergent process was scaled up accordingly. Samples were prepared in 50 mL BD Falcon™ tubes (BD Biosciences, CA, USA) and centrifugation was accomplished using a Beckman C6-6R centrifuge (Buckinghamshire, UK), operated at 3,000 rpm for 15 min. The XAD-4 step was also performed as a batch process and the separation of the sample form XAD-4 beads was by using either a solid-phase extraction column which retains the XAD-4 beads or by filtration via a 0.2 µm filter (Millipore, Watford, UK). Details of the detergent step performed at pilot-scale for the centrifugation validation study are provided in **Section 2.12**.

2.5 Ultrafiltration study comparing effects of Triton X-100 concentrations

The ultrafiltration (UF) study was performed using the Akta Cross Flow system (GE Healthcare, Amersham, UK) driving an ultra scale-down UF rig designed at UCL. In this study, the USD device was operated with 1.5 mL of feed material. Biomax disc membranes (Millipore, Watford, UK) with 100 kDa molecular weight cutoff and effective filtration area of 3.46 cm^2 were employed. Fresh membranes were used for

each study. Flux versus TMP curves were generated in total recycle mode, by recording steady-state permeate flowrates at gradual pressure increments. For all samples, viscosity was measured using a Brookfield Cone and Plate DVII+ Programmable Viscometer (Brookfield, Stoughton, MA) as described in **Section 2.13.11**.

2.6 PEG precipitation study

Equal volumes of cell lysate and PEG solutions prepared in 0.01 M sodium phosphate, pH 7.5, were added to achieve final PEG concentrations of 0% w/v to 25% w/v. The samples were allowed to incubate for 2 h with gentle agitation on a Eppendorf Thermomixer (Eppendorf, Cambridge, UK). The use of centrifugation and filtration were explored for the separation of the resultant precipitate from the mother liquor. For centrifugation, the samples were set up using 2 mL Eppendorf tubes and sedimentation of the precipitates was achieved via centrifugation at 12,000 rpm for 20 min (Eppendorf Centrifuge, 5415R, Cambridge, UK). For filtration, the samples were set up in 96-well Multiscreen filter plates (Millipore, MA, USA) and filtration was driven by centrifugation (Eppendorf Centrifuge 5810, Hamburg, Germany) at 900 rpm for 20 min. For precipitate resolubilisation, 0.01 M sodium phosphate buffer, pH 7.5, equalling the total volume of cell lysate and PEG solution at the start of precipitation was employed. To improve resolubilisation, a 0.2% v/v Triton X-100 buffer prepared in 0.01 M sodium phosphate, pH 7.5 was investigated.

2.7 Ammonium sulphate precipitation study

Equal volumes of cell lysate and ammonium sulphate solutions prepared in 0.01 M sodium phosphate, pH 7.5 were added to achieve final ammonium sulphate concentrations of 0% w/v to 25% w/v. The samples in eppendorf tubes were allowed to incubate for 2 h under gentle agitation on the Eppendorf Thermomixer (Eppendorf, Cambridge, UK) and subsequently centrifuged (Eppendorf Centrifuge, 5415R, Cambridge, UK) at 12,000 rpm for 20 min. The lipid precipitates formed a floatation layer above the soluble phase. The soluble phase was recovered by careful pipetting for analysis.

2.8 Investigating the selective product recovery methodology

The selective product recovery methodology is based on a twin centrifugation strategy. Following cell disruption by homogenisation, the yeast homogenate was diluted with 2 volumes of a 0.01 M phosphate buffer with a pH of 7.5 and the mixture was centrifuged using a Beckman C6-6R centrifuge (Beckman Coulter Inc., Buckinghamshire, UK) for 15 min at 3000 g to separate the cell solids from the cytosolic components. To investigate the partitioning of the HBsAg product and contaminants across the supernatant and solids fraction following centrifugation, both fractions were treated with a 0.01 M sodium phosphate buffer (pH 7.5) containing 0.6% v/v Triton X-100 of a volume equivalent to that used for the dilution of the yeast homogenate prior to the first centrifugation step. The detergent step was performed as described in **Section 2.4**. Material from the solids fractions was subjected to a second centrifugation step operated under identical conditions to the first centrifugation step for

the removal of contaminating solids. Prior to analysis, a Triton X-100 removal step using Amberlite XAD-4 was performed on all samples as previously described.

2.9 Investigating impact of homogenisation pressure on selective product recovery

Material was prepared for the homogenisation study as described in **Section 2.3**. The homogenisation study was performed on a Gaulin Micron Lab 40 (APV Gaulin GmbH, Lubeck, Germany). The samples were homogenised for 4 passes at various operating pressures between 100 to 1200 bar and a control sample of undisrupted cells was prepared. Temperature was maintained below 6 ^{o}C throughout cell disruption. The homogenate samples were subjected to the selective product recovery methodology as described in **Section 2.8** prior to analysis.

2.10 Investigating impact on hydrophobic interaction chromatography

Chromatographic separations were carried out using 1 mL Hi-trap Butyl-Sepharose® columns (GE Healthcare, Amersham, UK). Sample preparation involved adding to the samples 3 M ammonium sulphate solution in a 4:1 ratio to give a final concentration of 0.6 M. New columns were employed for each sample and the flowrate was maintained at 1 mL/min throughout the operation and separation was performed at

room temperature. For all samples, the columns were initially loaded with 2 mL of material in the equilibration buffer containing 20 mM sodium phosphate buffer with 0.6 M ammonium sulphate at pH 7. This was followed by washing using the same buffer to remove any unbound material. For elution, a buffer containing 10mM sodium phosphate at pH 7 was applied to the column. A second elution in 30% (v/v) isopropanol dissolved in 10 mM sodium phosphate at pH 7 was performed to desorb more tightly bound proteins. Fractions were collected at regular intervals with volumes of 0.3-0.5 mL during sample loading, early wash stage and product elution.

2.11 Ultra-scale down (USD) centrifugation study

The two key centrifugation operations in the selective product recovery methodology were evaluated independently based on the method described in **Section 2.8**. Feed material was prepared by homogenisation at 400 bar for 4 passes using the Gaulin Micron Lab 40 homogeniser (APV Gaulin GmbH, Lubeck, Germany). The centrifugation studies were performed using the Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany) and tempearature was maintained at 4 ^{o}C. To study the first centrifugation operation, pre-weighed Eppendorf tubes were filled with 1.5 mL of the homogenate and centrifuged for different lengths of time and at rotational speeds in the range of 3000 – 13,000 rpm to achieve Q/C$\sum$ conditions of 1.3 x 10^{-9} to 1.8 x 10^{-7} m/s in 6 replicates. A well spun sample used as the reference for solids recovery and dewatering was prepared by centrifugation for 40 min at 13,000 rpm to achieve a corresponding Q/C$\sum$ value of 1.1 x 10^{-9} m/s. All supernatant fractions were carefully removed by pipetting. Residual liquid and lipid layer remaining on the wall of the tubes

were removed with the aid of a tissue. The Eppendorf tubes containing wet cells were weighed. For each centrifugation condition, a set of 3 wet pellet samples were kept for detergent-mediated HBsAg liberation while the other 3 were left to dry in an oven overnight at 100 ^{o}C and reweighed after the drying process. Following the detergent step (described below), a second centrifugation step was performed for recovery of soluble product and this was at constant Q/C$\sum$ of 1.1 x 10^{-9} m/s for all samples.

To study conditions of the second centrifugation step, the first centrifugation step was performed for all samples at the optimal Q/C$\sum$ condition determined above. Following the discard of the supernatant from the first centrifugation step, Eppendorf tubes containing wet cells were weighed and treated with a phosphate buffer containing 0.6% v/v of Triton X-100 detergent as described previously in **Section 2.8**. The second centrifugation operation was carried out under a range of Q/C$\sum$ values (1.3 x 10^{-9} to 6.5 x 10^{-7} m/s) in 6 replicates. The supernatants from this second centrifugation step were recovered and the Eppendorf tubes containing the wet sediment weighed, left to dry and then reweighed.

2.12 Pilot-scale centrifugation study for USD validation

The pilot-scale centrifugation validation study was performed using the CARR Powerfuge^{TM} P6 to compare the use of the optimal conditions predicted from the USD studies (Batch A) against the worst case scenario (Batch B). Due to the lack of availability of recombinant *S. cerevisiae* expressing the HBsAg product, baker's yeast (Craftbake High Activity, DCL Yeast Ltd, Surrey, UK) was employed for this study. Material for the first centrifugation step was prepared as previously described in **Section**

2.8 at a 10L scale using diluted cell homogenate produced at 400 bar for 4 passes using the Gaulin Micron Lab 60 homogeniser (APV Gaulin GmbH, Lubeck, Germany). Temperature was maintained at 4 ^{o}C for all centrifugation studies. For Batch A, the first centrifugation step was performed at a feed flowrate of 1 L/min with a rational speed of 5,000 rpm ($Q/C\sum = 1.67 \times 10^{-7}$) while for batch B this was carried out at a feed flowrate of 0.1 L/min with a rotational speed of 15,000 rpm ($Q/C\sum = 1.9 \times 10^{-9}$). The solids fraction from both Batch A and B were recovered and resuspended with ~ 7 L of a 0.6% v/v Triton X-100 buffer and mixing was accomplished in a 20L LH 2000 Insitu Inceltech fermenter (Inceltech UK Ltd, Slough, UK) with mixing at 200 rpm at room temperature. The second centrifugation step was performed for Batch A at a feed flowrate of 0.1 L/min with a rotational speed of 15,000 rpm ($Q/C\sum = 1.9 \times 10^{-9}$) and for Batch B at a 1 L/min feed flowrate and 5,000 rpm in terms of rotational speed ($Q/C\sum = 1.67 \times 10^{-7}$). Samples of the supernatant and solid fractions from Batch A and B for both centrifugation stages were analysed to determine the levels of dewatering, solids recovery, clarification and the amounts of host protein and lipid contaminants present.

2.13 Analytical techniques

2.13.1 Optical density measurements

Optical density (OD) measurements were performed on a BioMate3 spectrophotometer (Thermo Spectronic, Rochester, NY, USA) at a wavelength of 600nm. Sarstedt cuvettes (Sarstedt, Numbrecht, Germany) were employed and the machine was blanked with DI water prior to analysis. The linear range for detection was

between 0 and 1 Au. Samples with OD values > 1 Au were diluted and re-measured. [Coefficient of variance from duplicate samples ≤ 5%.]

2.13.2 Dry cell weight analysis

Dry cell weight analysis was performed by filtering a known volume of sample through a pre-weighed Whatman Glass Microfibre Filter Grade GF/F (Whatman, Maidstone, UK) and rinsed with DI water. The filter was then dried overnight in an oven at 100 ^{o}C and re-weighed. Dry cell weight, which is a reflection of the cell density achieved in fermentation, was calculated as shown in the **Equation 2.1** below where DCW represents dry cell weight, F_f, mass of the filter containing cells before drying, F_o, mass of an empty filter and V, the volume of sample filtered through. [Coefficient of variance from duplicate samples ≤ 10%.]

$$DCW = \frac{F_f - F_o}{V} \qquad \text{(Equation 2.1)}$$

2.13.3 Glucose analysis

Offline glucose analysis was performed using a YSI bioanalyser (YSI Life Sciences, Yellow Springs, Ohio, USA). The method has a linear detection range from 0-5 g/L. Samples with glucose concentrations above this range were diluted accordingly prior to analysis. [Coefficient of variance from duplicate samples ≤ 2%.]

2.13.4 HBsAg determination by ELISA

HBsAg concentration was determined using the Murex HBsAg ELISA assay kit from Abbott Laboratories (Maidenhead, Beckshire, UK). Recombinant HBsAg (1 mg/mL) from Serotec (Oxford, UK) was used as the standard and calibration curve was generated from serial dilution of the standard. Due to the narrow linear range of the assay (linear range observed for concentrations below 3 x 10^{-4} mg/mL), samples were diluted 1000-fold with buffer (0.01 phosphate, pH7.5). This assay functions on a sandwich format whereby 75 µL of diluted samples were added along with 25 µL of sample diluent from the kit containing detergents and bovine and goat proteins to a 96-well plate coated with mouse monoclonal antibody to HBsAg. The plate was incubated for 1 h at 37 ^{o}C and following this, 50 µL of conjugate horseradish-peroxidase labeled goat antibody to HBsAg buffered in bovine and goat proteins were added to each well. A second incubation period of 30 min at 37 ^{o}C followed this. The plate was then rinsed 5 times using wash buffer of glycine/borate solution diluted 5-fold in RO water. Subsequently, 100 µL of a substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) was added and during this stage, for wells containing the HBsAg product, a colour change from pink to blue / purple would be observed. The plate was again incubated for 30 min at 37 ^{o}C. The enzyme-substrate reaction responsible for the colour change was quenched by the addition of 50 µL of 2 M sulphuric acid which resulted in a second colour change from blue / purple to yellow. Detection was performed using the Tecan Safire II microplate reader (Tecan Group Ltd, Mannedorf, Switzerland) at a wavelength of 450 nm using 690 nm as the reference wavelength. [Coefficient of variance for triplicate samples of ≤ 17%.]

2.13.5 Total protein assay by Bicinchoninic acid assay (BCA)

Total soluble protein was determined using a bicinchoninic acid assay (BCA) kit (Sigma, Dorset, UK.) Calibration was performed by 7 steps of serial dilution using bovine serum albumin (BSA) of 2 mg/mL which accompanied the kit to achieve a protein range from 0.15 – 2 mg/mL. Samples were diluted 2 – 8 fold depending on their starting concentration to ensure that the final concentrations fell within the linear detection range. 20 µL of sample or standard was added with 200 µL of BCA reagent on a 96-well plate (Sarstedt AG & Co, Leicester, UK or Nunc, Thermo Fisher Scientific, Leicester, UK). The samples were incubated at 37 ^{o}C for 30 min and a colour change from green to blue was observed for samples containing protein. Detection was performed using the Tecan Safire II microplate reader (Tecan Group Ltd, Mannedorf, Switzerland) at an absorbance wavelength of 562 nm. [Coefficient of variance for triplicate samples ≤ 10%.]

2.13.6 Lipid analysis by HPLC

Sample preparation for HPLC was carried out by adding 70 µL of sample to 930 µL sample buffer containing chloroform, TFA in methanol (TFA:methanol = 1:285) and methanol in 50:3:40 v/v ratio. The mixture was then centrifuged at 13,000 rpm for 2 min to remove precipitated proteins from solution. Supernatant containing soluble lipids was decanted into vials for HPLC analysis. The HPLC lipid assay used a Jordi Gel Glucose DVB 500 column (Alltech, Deerfield, IL, USA) and operated at a flowrate of 1 mL / min, with a run time of 25 min under an isocratic mode of separation. Absorbance was monitored at 254 nm to detect for ergosterol which is the major lipid contaminant in yeast cells (Veen & Lang, 2004). Running buffer contained chloroform, methanol

and 0.15% TFA in water at a ratio of 50:42:7 (v/v). Ergosterol standard of 10 µg/mL was analysed in order to identify the major lipid peak. The minor peaks, however, were unidentifiable and to account for these, results were presented in terms of total peak area. [Coefficient of variance (for peak area) for duplicate samples ≤ 4%.]

2.13.7 Protein analysis by size exclusion chromatography (SEC)

Samples of 100 µL were loaded onto a Superose 6 10/300 GL column (GE Healthcare, Amersham, UK) which was operated at 0.5 mL/min for a total run time of 90 min using 0.5 M phosphate running buffer, pH 7. Absorbance was monitored at 280 nm. Protein profiling using this method was based on the elution time of the peaks with larger proteins eluting earlier. [Coefficient of variance for peak area for duplicate samples ≤ 4%.]

2.13.8 Nucleic acid determination

Prior to analysis, the nucleic acids were extracted using a QIAprep kit (Qiagen, West Sussex, UK), following the protocol accompanying the kit. Nucleic acid determination was carried out using a NanoDrop® ND-1000 (NanoDrop®, Delaware, USA) where the measurements of nucleic acid contents are determined by the software based on the absorbance measurements of the samples at 260 nm. [Coefficient of variance for triplicate samples ≤ 10%.]

2.13.9 VLP fractionation by size exclusion chromatography for particle size analysis

Samples filtered using a 0.2 µm hydrophilic filter (Millipore, Watford, UK) were loaded onto a Superose 6 10/300 GL column (GE healthcare, Amersham, UK). Separation was carried out at a flowrate of 0.5 mL / min for a total run time of 90 min. The running buffer was 0.5 M phosphate buffer, pH 7. Fractions of 1 mL were collected and the active peak identified using an ELISA based method detailed in **Section 2.13.4**.

2.13.10 Particle size analysis by dynamic light scattering

Particle size analysis was carried out using a Malvern Zetasizer Nano S (Malvern Instruments Ltd., Worcestershire, UK) which operates based on dynamic light scattering. This equipment allows size measurements in the nanometer range. Prior to sample analysis, 70 µL of samples were filtered through a 0.2 µm hydrophilic filter (Millipore, Watford, UK). Data presented are based on particle size / volume distribution. DLS volume distribution was chosen over intensity distribution since the latter is more susceptible to signal domination by large sized particles which may provide misleading representation of the population, and DLS volume distribution was favoured over number distribution to minimise the error in data conversion (User Guide, Malven Instruments). Samples were measured in triplicate and the number of scans taken was set to automatic (instrument determines the number of measurements to achieve good quality data taking into account sample polydispersity) and the measurement errors are denoted on the histogram generated by the software, showing an average coefficient of variance of 10%.

2.13.11 Viscosity measurement

Viscosity measurements were performed using a Brookfield Cone and Plate DVII+ Programmable viscometer (Brookfield, Stoughton, MA, USA). The machine was initially autozeroed and a check was performed to ensure the pin on the cone and cup were correctly positioned by running the machine at 10 rpm and monitoring the display for jumps or unsettled readings. A sample of 1-2 mL was then added to the chamber and the shear stress and shear rate measurements were recorded at a range of rotational speeds of 40-200 rpm. Shear stress values were then plotted against shear rates and the viscosity of the sample determined by the slope of the stress-strain relationship. A control was performed using RO water. [Average coefficient of variance $\leq 10\%$]

2.13.12 Microscopy analysis

Cell homogenate generated at different pressures together with the undisrupted sample as a control, were studied under a Leica DM light micrcoscope (Leica Microsystems Ltd, Heerbrigg, Switzerland). For sample preparation, 10 µL material was added to a microscope slide and a cover slip was lowered gently to avoid the formation of air bubbles. Initial focusing was carried out at 400 X magnification using the 40 X objective. For 1000 X magnification, oil was added to the cover slip for immersion using the 100 X objective. The software on the Leica system allows the microscope images to be captured and saved. A scale was drawn using the Image J programme (National Institute of Health, USA, 1997) based on the correlation of number of pixel to length information from the Leica and this provided a capacity to record the key dimensions of the visible cell homogenate components.

2.13.13 Analysis of solids recovery or clarification level

For centrifugation studies, Eppendorf tubes were weighed initially empty and at the end of the experiment when they contained solid fractions dried in an oven at 100 ^{o}C overnight. The difference in weight equals the dry cell weight. A reference for 100% (w/w) solids recovery was setup using a well spun sample prepared by centrifugation at 13,000 rpm for 40 min ($Q/C\sum$ = 1.1 x 10^{-9} m/s). For all other samples, the solids recovery level were calculated proportional to the reference data as shown in **Equation 2.2**. [Average coefficient of variance ≤ 10%.]

$$\%\ solids\ recovery = \frac{Dry\ cell\ weight\ of\ sample}{Dry\ cell\ weight\ of\ reference}\ \text{x}\ 100\% \qquad \text{(Equation 2.2)}$$

2.13.14 Analysis of dewatering level

The dry cell weight was measured as above, determined as the difference between the weights of the Eppendorf tubes when containing wet solid fractions and when empty. A well spun sample prepared under the conditions described in **Section 2.11** was used as the reference and denoted as 100%. The ratio of the dry cell weight to the wet cell weight (dw_r) was calculated and this constant used to account for the amount of intracellular water removed by the drying process (Salte *et al.*, 2006). Percentage dewatering for all samples can be calculated as shown in the **Equation 2.3** below whereby WW_{sed} and DW_{sed} respectively denote the wet and dry weights of the sedimented cells. [Average coefficient of variance ≤ 10%.]

$$\%\ dewatering = 100\% - \left[\frac{WW_{sed} - \frac{DW_{sed}}{dw_r}}{WW_{sed}}\ \text{x}\ 100\%\right] \qquad \text{(Equation 2.3)}$$

2.14 Application

This chapter provides a compilation of the main experimental and analytical procedures in this research and serves as a point of reference for discussions in the following results chapters on materials and experimental methods employed. Specific modifications to the procedures reported here for individual studies, if any, are described in the corresponding results chapters.

Chapter 3

Fermentation Development

3.1 Introduction

Saccharomyces cerevisiae is an attractive host strain for the large-scale expression of recombinant proteins as it is non-pathogenic for humans, relatively free of endotoxins and has favourable fermentation characteristics such as high protein productivity, reproducibility and amenability to scale up (Carty *et al.*, 1989; Buckland, 2005). Yeast cells have the added ability of performing complex eukaryotic-like post-translational modifications producing proteins similar to those of the mammalian origin. A wealth of genetic information on *S. cerevisiae* is available and this facilitates the engineering of the desired cell metabolism and expression characteristics using this strain.

The hepatitis B surface antigen (HBsAg) from Merck & Co Inc. (West Point, PA, USA) examined in this study is expressed recombinantly in *S. cerevisiae*. For plasmid maintenance and to confer a competitive advantage over wild type cells, the plasmid containing the HBsAg gene has also been inserted with a Leu+ gene (Carty *et al.*, 1989). This allows the use of a leucine-free selective media for fermentation in which the recombinant strain capable of synthesising the amino acid leucine would thrive whilst the growth of wild type cells would be hindered.

The HBsAg gene is expressed under the control of the GAL-10 promoter (Fu *et al.*, 1996) and is regulated by the levels of glucose and galactose present during fermentation (St. John and Davis, 1981). When glucose becomes depleted during cell culture, the cells switch to galactose metabolism which induces the HBsAg gene and the product is synthesised. Composition of the culture media and feeding strategy has an enormous impact on cell growth and protein expression characteristics. Glucose is employed as the main carbon source for HBsAg fermentation however high glucose levels in yeast could cause catabolite repression which impairs cell growth. A synthetic medium containing glucose but with minimal catabolite repression effects was developed by Oura (1974). Galactose is another vital media component in fermentation for the regulation of the HBsAg gene. The interactions between glucose and galactose in the regulation of HBsAg expression has been reported (Carty *et al.*, 1989). Galactose could be added when glucose has been depleted for time-based induction which could minimise the effects of catabolite repression due to the use of overly rich media (Gancedo, 1998) or at the start of fermentation for auto-induction which serves to reduce process monitoring requirements.

In this study, the master cell bank of the recombinant yeast cells was donated by Merck & Co. Inc (West Point, PA, USA). To generate material for purification studies, fermentation was carried out at pilot-scale at the Advanced Centre for Biochemical Engineering at UCL. Although fermentation itself was not the focus of this thesis, the production of a realistic feed stock for subsequent purification studies was critical.

This chapter therefore describes fermentation development studies performed to determine the suitable media composition and feeding strategy for the recombinant *S. cerevisiae* cells. Two different fermentation media compositions were investigated: (1) a fully defined media developed by Yau (2005) which was adapted from previous

studies by Oura (1974) and Fu *et al.* (1996) and has been successfully demonstrated for wild type yeast fermentation; and (2) a semi-defined media reported by Joyce *et al.* (1998) for the production of the human papillomavirus (HPV) vaccine from recombinant *S. cerevisiae*. Trial fermentations at 20L scale was initially performed using wild type *S. cerevisiae* to provide an understanding of growth characteristics and requirements of yeast cells. For the recombinant strain, a similar protocol was adopted but with modifications in terms of the media composition, galactose addition time and the length of fermentation. Fermentation performance was evaluated based on cell growth characteristics as indicated by dissolved oxygen levels, glucose consumption, cell density measurements and the HBsAg productivity levels targeted at 100 mg/L based on Merck process recommendations. The suitable media composition and feeding strategy were then scaled up to 75 L to generate material for the subsequent HBsAg purification studies.

3.2 Materials and Methods

Fermentation development studies were performed using two different strains of *S. cerevisiae*. A wild type *S. cerevisiae* strain, ATCC 26786 (ATCC, Middlesex, UK) was initially employed for a trial fermentation and subsequent studies were performed using the recombinant *S. cerevisiae* strain (Merck & Co. Inc, West Point, PA, USA) which produces the HBsAg product. Two different fermentation media were evaluated for HBsAg production and the media composition are detailed in **Section 2.1.2**. Cell banking methods were described in **Section 2.1.3**. Fermentation experiments were conducted initially at a 20 L scale and the developed process was subsequently scaled

up to 75 L as reported in **Section 2.1.4**. The pilot-scale process employed for the production of HBsAg feed stock for purification studies is described in **Section 2.2**.

Analytical techniques for fermentation process monitoring include online measurements of pH, temperature, dissolved oxygen tension (DOT), agitation speed and air flow rate (**Section 2.1.4**), determination of cell density by optical density measurements (**Section 2.13.1**) and dry cell weight analysis (**Section 2.13.2**), measurement of glucose levels (**Section 2.13.3**) and determination of HBsAg productivity via an ELISA assay (**Section 2.13.4**).

3.3 Results and Discussions

Fermentation studies were carried out to develop a suitable protocol for the production of a realistic feed stock for HBsAg purification research. Initial studies were carried out at 20 L and the developed process was subsequently scaled up to 75 L. The media previously reported by Yau (2005) and the Joyce *et al.* (1998) were assessed in terms of their ability to support growth of recombinant *S. cerevisiae* cells and HBsAg productivity titres that are representative of the industrial process.

3.3.1 Wild type *S. cerevisiae* fermentation in the Yau (2005) media

A trial fermentation was carried out using wild type *S. cerevisiae* from ATCC with the aim of gaining better understanding on yeast cell growth characteristics and media requirements. The fermentation was performed at 20 L scale with 12 L working volume using the Yau (2005) media which was developed based on previous works by

Fu *et al.* (1996) and Oura *et al.* (1974). 20 g/L glucose was added at the start of fermentation as the main carbon source and when this had depleted, galactose was added to induce HBsAg production.

The fermentation profile recorded is as shown in **Figure 3.1**. A drop in the dissolved oxygen tension (DOT) was observed from the start of fermentation due to increased oxygen consumption which is indicative of increased cell growth rate. The DOT level plateaued at the minimum DOT set point of 30% maintained by the feedback regulation of the agitation rate to compensate any further increase in oxygen consumption. At approximately 18 hours, a rise in the DOT level was detected suggesting a decrease in the cell growth rate. Off-line glucose measurement indicated that the glucose level was near depletion at this stage. Galactose was added for induction. A dip in the DOT level was observed at approximately 25 hours and this is likely due to the switch to galactose metabolism.

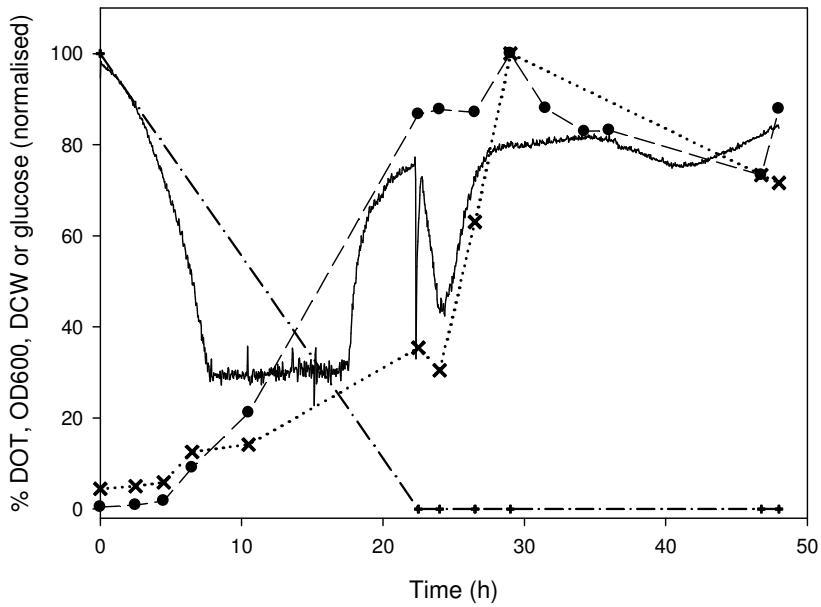


***Figure 3.1**: Fermentation profile obtained for wild type* S. cerevisiae *grown in media adapted from Oura (1974) and Fu et al. (1996) showing variations in DOT (———), OD600 (—•—) dry cell weight (DCW) (·····×·····) and glucose levels (—·+·—) over the culture period.*

Offline measurements of OD600 and dry cell weight followed the trends in the DOT profile. D600 and dry cell weight plateaued after approximately 20 hours when the glucose supply had been depleted and galactose metabolism begun. The fermentation lasted 48 hours achieving a maximum OD600 of 27 Au and maximum dry cell weight of 22 g/L.

The results from the wild type *S. cerevisiae* fermentation showed that the adapted media could support cell growth and may be employed for the production of HBsAg. Assuming that the growth characteristics of the recombinant strain were similar to the wild type, glucose level would be expected to deplete after ~ 23 hours after which galactose induction would commence. Subsequent studies were performed with the recombinant strain to verify this.

3.3.2 Recombinant *S. cerevisiae* fermentation using the Yau (2005) media

Following from the promising fermentation performance of the wild type *S. cerevisiae* cells, the Yau (2005) media was investigated for culturing the recombinant strain. During the seed stage, low OD600 measurements were observed. After 24 hours of culture OD600 of < 0.3 Au was achieved in comparison to the wild type strain which showed OD600 > 1 Au at the same time point. OD600 values correlate to the number of cells detected. Hence the low OD600 values recorded could be due to poor cell growth or increased cell aggregation due to the *mnn* mutation in yeast which renders the cells clumpy during growth (Mendez-Vilas *et al.*, 2004). A reddish tint was also detected in the shake flask cultures. This may have been caused by an *ade1* mutation which leads to the accumulation of an adenine precursor which later polymerises into a pigment (Ugolini & Bruschi, 1996; Crowley & Kaback, 1984).

Despite the low OD levels, the shake flask cultures were used to inoculate a 20 L fermenter. Galactose was added together with glucose at respective concentrations of 20 g/L at the start of fermentation for auto-induction and the culture was maintained for ~ 90 hours. The DOT level decreased only slightly and remained above 80% over the fermentation period. The low oxygen consumption indicated that the rate of cell growth was low. A maximum OD600 of 7 Au and dry cell weight of 8 g/L were obtained but only towards the end of the ~ 90 hour culture period. Offline glucose analysis by HPLC further confirmed that cell metabolism was slow. For the wild type fermentation, the glucose level was depleted after ~ 20 hours, however this did not occur until after 60 hours for the recombinant strain investigated here. Due to the poor growth characteristics and the late galactose induction (after 60 hours), a low HBsAg titre of ~ 12 mg/L was measured.

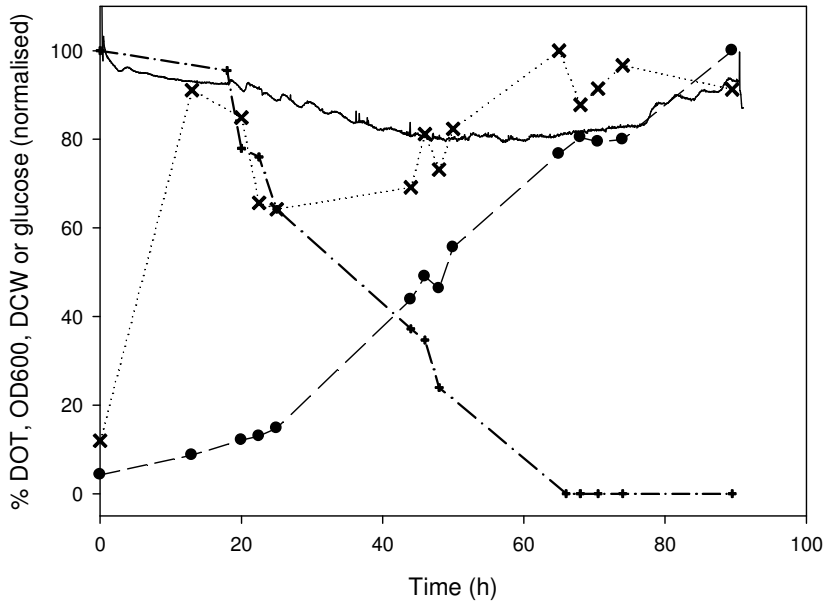


***Figure 3.2**: Fermentation profile obtained for recombinant* S. cerevisiae *grown under the same conditions and media composition as the wild type strain showing variations in DOT (———), OD600 (—•—), dry cell weight (DCW) (···×···) and glucose levels (—·+·—) over the culture period.*

It is evident that the media previously shown to be successful for the wild type fermentation did not achieve the same cell growth characteristics for the recombinant strain. Galactose addition at the start of fermentation for auto-induction could have contributed to the poor cell growth observed, hence further studies to investigate this would be required if such a media were to be used as the basis for large scale HBsAg production.

In view of the poor cell growth observed using the Yau (2005) media, a second media originally developed for the large scale production of the HPV VLP vaccine (Joyce *et al.*, 1998), was investigated for HBsAg production.

3.3.3 Recombinant *S. cerevisiae* fermentation in media from Joyce *et al.* (1998)

The fermentation protocol described in Joyce *et al.* (1998) originally for producing the HPV vaccine employs a culture media which is semi-defined and leucine free both for the seed stages and for HBsAg production. An improvement in cell growth over that achieved with the earlier Yau (2005) media was observed during the seed stages. After a 24-hour period, OD600 values of > 2 Au were attained and the cultures did not show the reddish colouring previously observed for the Yau (2005) media.

Fermentation for HBsAg production was carried out also with additions of 20 g/L glucose and 20 g/L galactose for auto-induction. The fermentation profile is shown in **Figure 3.3**. A rapid drop in the DOT profile was observed at the start of fermentation reaching the minimum DOT set point of 30% at ~ 10 hours at which point the DOT plateaud at this level for a further 20 hours. The DOT profile was similar to that obtained for the trial fermentation with the wild type strain in which high oxygen

consumption rates indicated healthy cell growth. Off-line glucose measurements suggested that galactose induction occurred before ~ 22 hours which again was similar to that from the wild type fermentation.

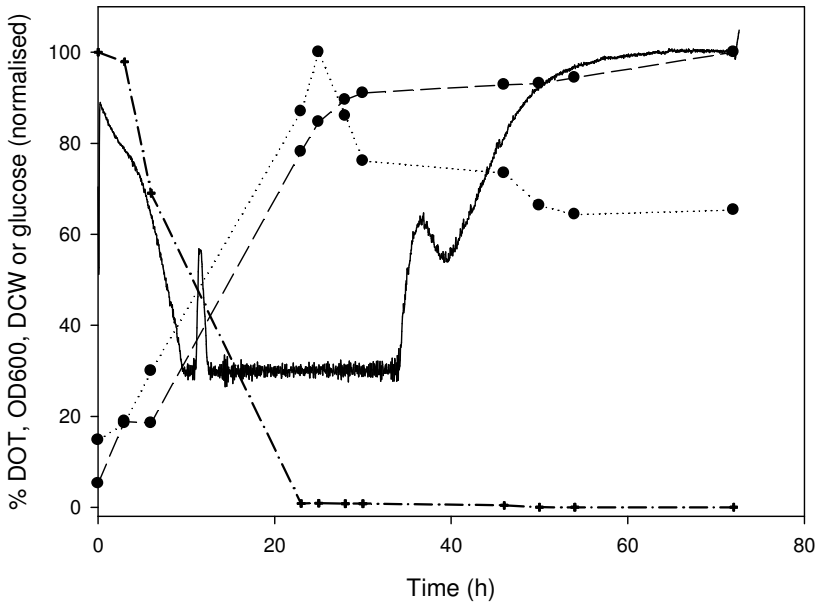


Figure 3:3*: Fermentation profile obtained for recombinant* S. cerevisiae *grown in the Joyce et al. (1998) media originally for HPV production. Variations in DOT (———), OD600 (—•—), dry cell weight (DCW) (·····×·····) and glucose levels (—·+·—) are shown over the culture period.*

Fermentation was carried out for a total of 72 hours achieving a maximum OD600 of 5.5 and maximum dry cell weight of 6 g/L which was slightly lower than the 9.5 – 9.7 g/L range reported by Joyce *et al.* (1998). Although the maximum OD and dry cell weight were similar to those from the Yau (2005) media for the recombinant strain, these values were achieved earlier at ~ 22 hours. This was beneficial as high cell density achieved earlier in fermentation would increase the productivity level of HBsAg over the culture period. ELISA assay results showed that an overall HBsAg titre of ~ 60

mg/L was attained from this fermentation, significantly closer to the range expected for this Merck strain.

3.3.4 Scale up of recombinant *S. cerevisiae* fermentation to 75 L scale

Based on the promising fermentation performance using the Joyce *et al.* (1998) media, the fermentation was scaled up in terms of volume, from 20 L to 75 L and in the supply of glucose and galactose which were doubled to 40 g/L respectively. The fermentation profile obtained is as shown in **Figure 3.4**. From the start of fermentation, a rapid decline in the DOT level to a minimum of ~ 40% was observed. Unlike that observed at the 20 L scale, the DOT level did not plateau but instead increased after the initial drop. [Further studies may be required to investigate if this was due to differences in oxygen transfer characteristics which are related to the design of the fermenters but these aspects were deemed to be beyond the scope of this thesis].

The scaled up process was maintained for an equal length of culture time of 72 hours. In spite of the higher supply of glucose at the start of fermentation, glucose depletion and galactose induction occurred around the same time point as of the 20 L batch. Maximum OD600 and dry cell weight measurements of ~ 6 respectively were similar to those previously attained however higher HBsAg titres were achieved with a maximum of 100 mg/L recorded at ~ 48 hours. Off-line HBsAg measurements over the course of fermentation are shown in **Figure 3.5**. The drop in HBsAg levels towards the end of fermentation corresponded to the decrease in dry cell weight measurements and may suggest that some level of cell death and proteolysis might have occurred. Based on this, the optimal harvest point would be ~ 50 hours to ensure that maximum HBsAg yield is achieved.

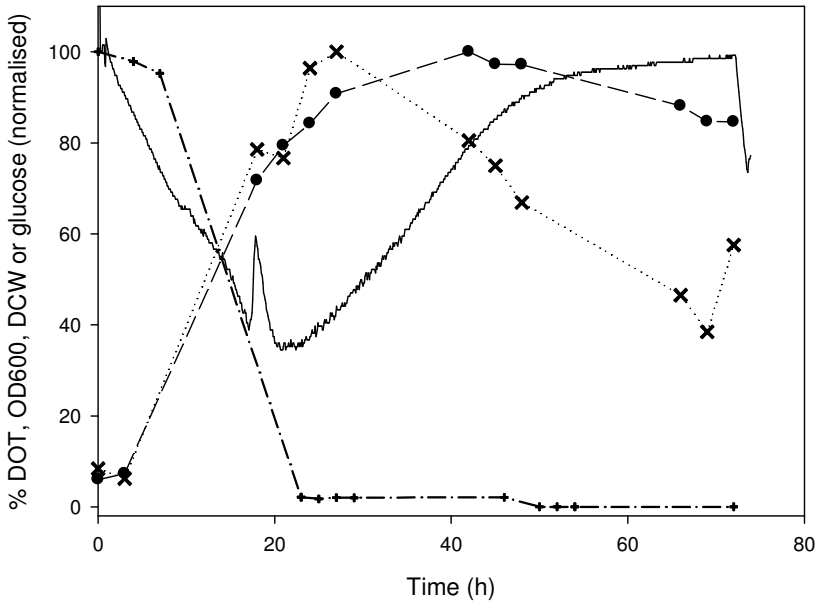


***Figure 3.4**: Recombinant* S. cerevisiae *fermentation scaled up to 75 L using the Joyce et al. (1998) media with 40 g/L glucose and galactose additions. Profiles of DOT (——), OD600 (—•—), dry cell weight (DCW) (·····×·····) and glucose levels (—+—) are shown over the culture period.*

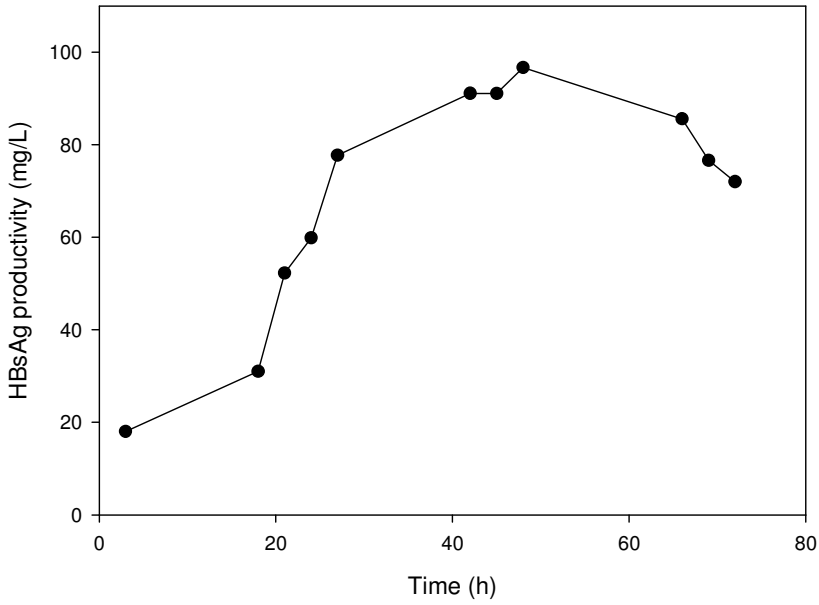


***Figure 3.5:** HBsAg expression per litre of fermentation broth measured off-line for the recombinant* S. cerevisiae *fermentation at 75 L scale with 2-fold increase in additions of glucose and galactose over the 20 L fermentation.*

A total of ~ 400 g of wet cells were harvested from this fermentation with an estimated total HBsAg yield of 5 g. The cell biomass from fermentation based on a maximum dry cell weight of 6 g/L and the HBsAg productivity level of 80-100 mg/L broth are within the target values expected from an industrial process. Based on this, it was deemed that the 75 L scale process demonstrated here was capable of generating feed stock that was sufficiently realistic for subsequent purification studies.

3.4 Conclusions

The HBsAg protein is recombinantly expressed in *S. cerevisiae* and to generate sufficient material for purification studies, the development of a suitable fermentation method was critical. Although fermentation itself was not the focus of this research project, it was important that the feed stock generated was representative of that obtained from an industrial process to ensure that results from subsequent purification studies were relevant. Fermentation performance can be assessed based on the cell biomass and HBsAg productivity levels achieved. Fermentation developmental studies were performed at 20 L scale based on two previously reported fermentation media compositions. The first was a defined media developed by Yau (2005) based on previous studies by Oura (1974) and Fu *et al.* (1996). Although this media had shown promising results for wild type *S. cerevisiae* fermentation, the rate of cell growth was very low when used for the recombinant HBsAg producing strain. This was reflected by the low oxygen consumption levels as DOT measurements remained > 80% over the cell culture period and the low final HBsAg productivity titre. The second media investigated was originally developed by Merck & Co. Inc for the production of the

HPV vaccine in recombinant *S. cerevisiae* (Joyce *et al.*, 1998). Fermentation using this media for the production of HBsAg was successful. Healthy cell growth was evident from the oxygen consumption and cell density profiles. Significantly higher HBsAg titre was achieved. This fermentation was scaled up to 75 L and the supply of glucose and galactose were doubled. Similar cell growth characteristics and an improvement in HBsAg productivity titre were observed upon scale up. The cell densities and HBsAg titres were comparable with those achieved in industry (Joyce *et al.*, 1998). Having fulfilled these criteria the scaled up process was deemed suitable for the production of HBsAg for purification studies which formed the next stage of the investigations.

Chapter 4

Detergent-mediated HBsAg liberation

4.1 Introduction

Although production of HBsAg has been achieved using bacteria, transgenic plants (Kumar *et al.*, 2005) and mammalian cells (Diminsky *et al.*, 1997), expression in *S. cerevisiae* remains an attractive option. Yeast fermentation is well established, highly reproducible and amenable to large scale operation (Buckland, 2005). However, as previously discussed, the HBsAg is a lipid-envelope protein and its expression using *S. cerevisiae* as the host cell could raise specific challenges in the recovery of the product. The p24s monomers of the HBsAg are secreted cotranslationally into the endoplasmic reticulum (ER) and bud into the lumen as 20-nm lipoprotein particles using host lipid (Eble *et al.*, 1986). Since the protein is permanently localised within the ER (Biemans *et al.*, 1992), cell disruption using high pressure homogenisation followed by a detergent step is necessary to release the VLP from the tightly associated membrane components. The multistage process for the recovery and purification of HBsAg from yeast cells is shown in **Figure 4.1**. Following release of HBsAg into the process stream, cell membrane debris and other contaminating solids are removed by centrifugation. Subsequently, detergent is removed from the process stream by recirculation of the material through an XAD-4 column. Purification of HBsAg is typically achieved through a series of chromatographic stages (Dekleva, 1999; Belew *et al.*, 1999)

although the use of precipitation and density ultracentrifugation (Deml *et al.*, 1999) has also been reported.

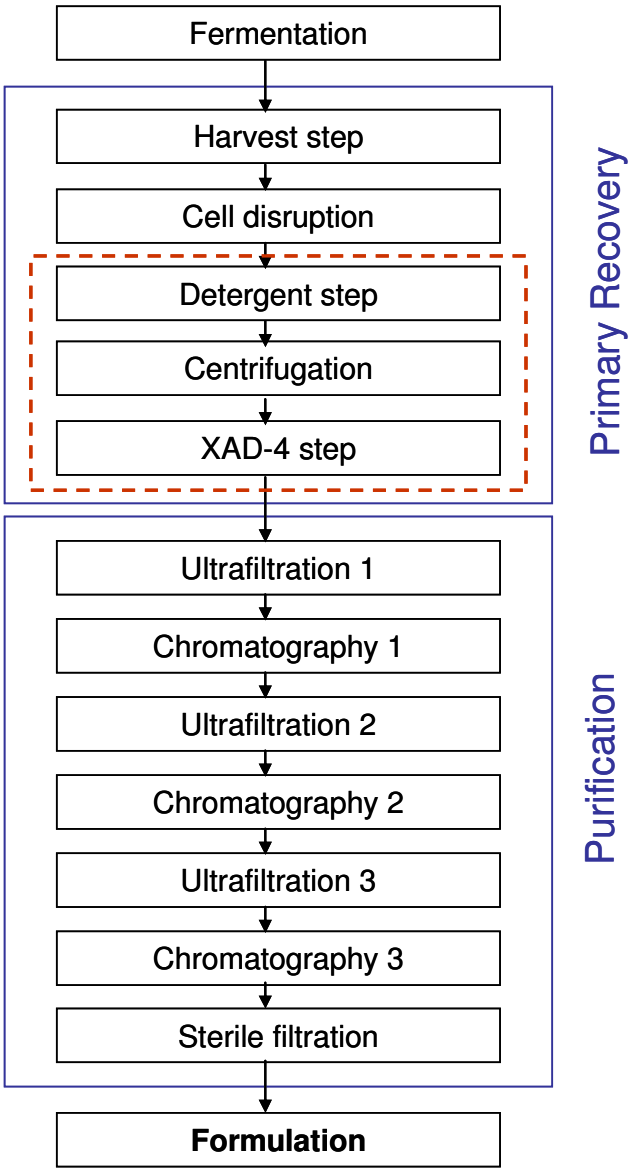


***Figure 4.1**: Typical process flowsheet for recovery of HBsAg from* S. cerevisiae. *(Adapted from Dekleva, 1999). (---) highlights unit operations focused on in this study.*

Detergent-mediated liberation of VLP is a critical step in the recovery of HBsAg. Detergent facilitates recovery of HBsAg by weakening interactions that exist between the VLP and membrane components (Chi *et al.*, 1994) and by promoting separation of yeast cell membrane from other unwanted cellular debris (Sitrin and Kubek, 1992), allowing better access of the detergent to the ER. A study by Smith and co-workers on extraction of HBsAg from recombinant plant systems further suggested that detergent may also promote VLP vesicle formation by driving the inward budding of the ER membrane (Smith *et al.*, 2002; Kreibich *et al.*, 1982)

Based on the above, it is likely that increases in detergent concentration would favour VLP recovery. However, it may also be necessary to take into consideration the fact that the HBsAg particle is essentially a lipoprotein, composing of 75% protein and 25% lipid by weight (Gavilanes *et al.*, 1982). The lipid moiety has been shown to perform critical roles in the maintenance of native structure and antigenic activity (Gavilanes *et al.*, 1990; Sevcsik *et al.*, 2007). Excess detergent could have detrimental effects on the lipid constituents of the HBsAg particle leading to a decrease in immunogenic activity.

The yeast endoplasmic reticulum and other membranous organelles are rich in lipid and protein. Lipid production in yeast cells increases during extended cell cultures (Wijnendaele *et al.*, 1987), which is necessary in the production of VLPs to achieve the desired protein expression levels. It is likely that the addition of detergent would promote the release of membrane associated host proteins and lipids and that the level of these contaminants would be a function of detergent concentration. Any increase in the extent of host lipid and protein contamination would consequently have implications for the performance of subsequent downstream operations.

Triton X-100 is the most commonly employed detergent for the liberation of HBsAg from the ER although the use of other detergents such as variants of Triton-X, CHAPS and polysorbate (PS) and glycol ether solvents has been reported for similar applications (Wijnendaele *et al.*, 1987; Kniskern and Hagopian, 1992; Allen *et al.*, 2007). [Properties of these detergents were discussed in Section 1.5.1.] For Triton X-100, there is also no consensus as to the level of detergent requirements reported. For example, Wampler *et al.* (1985) treated cell lysate with 4 volumes of 0.1% v/v Triton X-100 solution whereas Sitrin & Kubek (1991) proposed a concentration of 0.5% v/v. The different amounts of detergent reported could be due to variations in the process and linked to differences in cell culture and cell disruption conditions.

This chapter aims to investigate the influence of the detergent step on key process parameters in order to facilitate the development of a framework which could benefit the process design of future generation lipoprotein vaccines. A detergent screen was initially carried out to compare the performance of Triton X-100 to other commonly employed detergents and chemicals to determine if indeed Triton X-100 was the superior agent for HBsAg liberation. Having determined the most suitable detergent for this purpose, its effects on HBsAg recovery were analysed at three different levels: (1) the direct impact of the amount of detergent employed on the level of HBsAg recovery and product quality, (2) characteristics of the process streams resulting from the use of different detergent concentrations in terms of the level of host lipid and protein contamination, and (3) the indirect impact of the detergent step on the performance of the ultrafiltration step immediately following primary recovery. The results from this study would assist in the identification of best trade-off conditions for HBsAg recovery which can be adopted as a reference point when designing the process for future generation vaccine candidates.

4.2 Materials and Methods

The experiments reported in this chapter were performed using HBsAg feed stock generated from fermentation as detailed in **Section 2.2** and disrupted by homogenisation as described in **Section 2.3**. The primary recovery process adopted for the screening studies to compare chemical agent for HBsAg liberation and subsequently to determine the optimal concentration range are discussed in **Section 2.4**.

The study to characterise the impact of detergent concentrations during the HBsAg liberation step on the performance of a downstream ultrafiltration operation is described in **Section 2.5**. Viscosity measurements to support the analysis of mass transfer resistance during ultrafiltration were conducted as described in **Section 2.13.11**.

Analyses performed for HBsAg particle characterisation and the profiling of contaminants in the product stream include an ELISA assay to measure HBsAg antigenicity (**Section 2.13.4**), HBSAg particle size measurements by dynamic light scattering (DLS) (**Section 2.13.10**), total protein concentration determination by a Bicinchoninic (BCA) assay (**Section 2.13.5**) and lipid analysis by HPLC (**Section 2.13.6**). Sample fractionation by size using size exclusion chromatography was carried out prior to DLS analysis as reported in **Section 2.13.9.**

4.3 Results and discussions

The objective of this study was to identify optimal conditions for detergent-mediated HBsAg liberation, which could be adopted in the process design for future generation lipid-envelope VLP vaccines. In this study, the criteria chosen for

determining optimal conditions for the detergent step include VLP yield, product quality and the level of host cell protein and lipid contaminants in the resulting process stream. Since VLP vaccines are to be administered to healthy patients, product quality is of paramount importance. Step yield and the degree of contamination are also considered since these parameters could have implications for the overall throughput and production costs.

4.3.1 Investigating the efficiency of a range of chemicals for HBsAg liberation

Although Triton X-100 is most commonly employed agent for the recovery of HBsAg following cell disruption, other detergents and solvents have been reported for similar applications (Wijnendaele *et al.*, 1987; Kniskern and Hagopian, 1992; Allen *et al.*, 2007). Hence, the first set of studies was performed to assess if indeed Triton X-100 was optimal for this purpose. The effectiveness of Triton X-100 for the liberation of HBsAg from the ER was compared to that of Polysorbate (PS) 20 and 80, Triton X-101, CHAPS and ethyl butyl ether (EB). The maximum achievable HBsAg recovery levels within the typical operating range of 0 – 1% w/v for the detergents (Wijnendaele *et al.*, 1987; Kniskern and Hagopian, 1992) and 0 – 30% w/v for EB (Allen *et al.*, 2007) were determined by an ELISA assay and are shown in **Figure 4.2**. The results demonstrate that the highest HBsAg yield was obtained using Triton X-100 thus verifying that this detergent was optimal for HBsAg recovery.

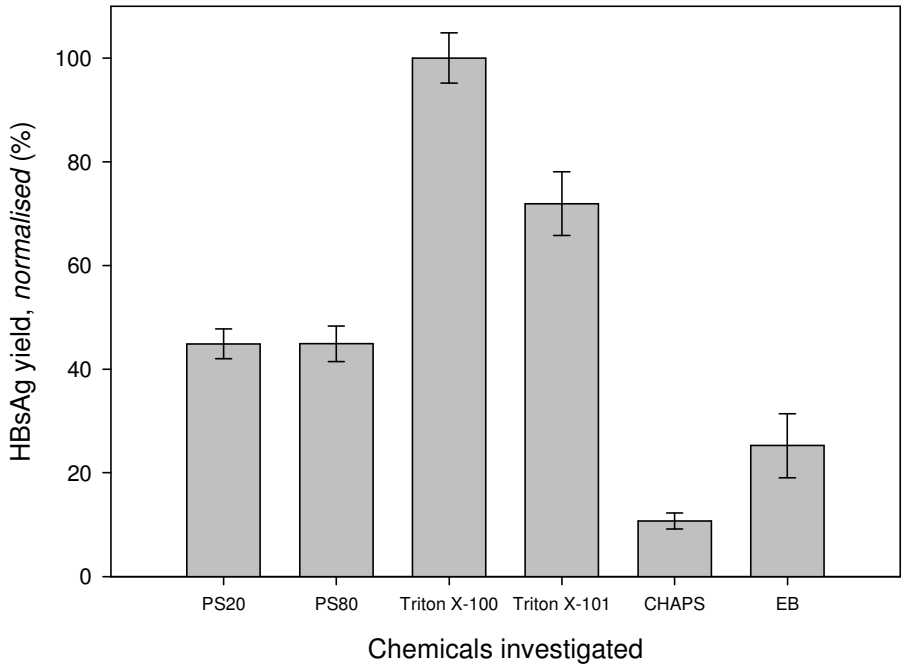


Figure 4.2: *Comparison of the HBsAg yield obtained from the use of different agents for the extraction of HBsAg product from the ER. The data has been normalised against the highest HBsAg yield measured (~18 mg HBsAg / g cell) and the error bars represent the standard deviation from triplicate samples.*

4.3.2 Investigating impact of Triton X-100 concentration on HBsAg recovery & activity

Having determined that indeed Triton X-100 was superior for the recovery of HBsAg, subsequent studies were performed to characterise the potential impact of the operating concentrations on the level of HBsAg recovery and product antigenicity. Different concentrations of the detergent were screened. An ELISA assay was again employed to measure the level of HBsAg activity in the samples at the end of primary recovery and the results are as shown in **Figure 4.3.**

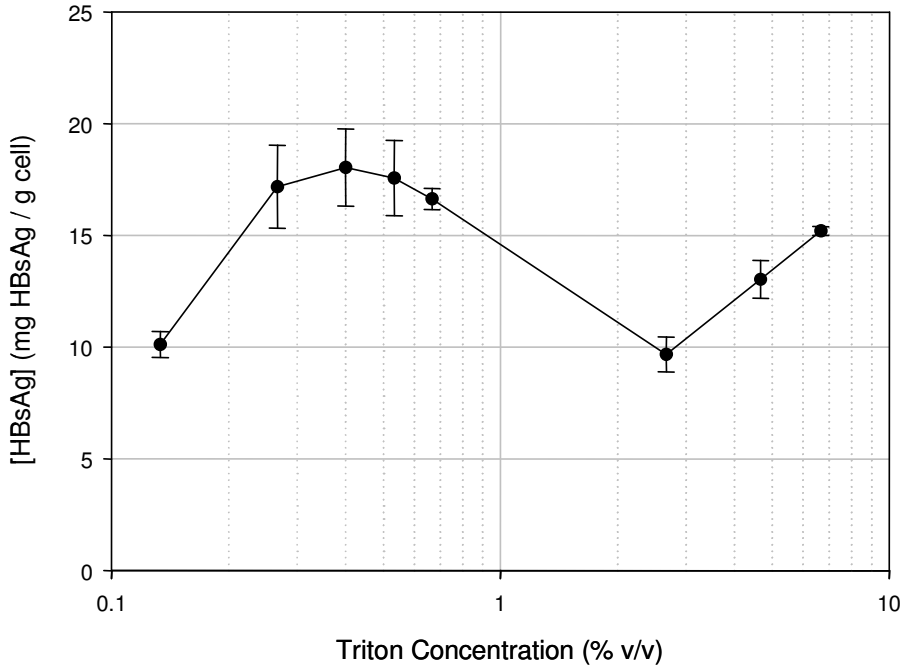


***Figure 4.3**: Recovery of HBsAg per gram of wet cell under different Triton X-100 concentrations determined by an ELISA activity assay. Negative control with no Triton X-100 gave a recovery level of 1.7 mg HBsAg / g cell. Error bars correspond to standard deviation from triplicate samples.*

Improvement in HBsAg activity in the product stream was observed when Triton X-100 concentrations were increased from 0% to 0.5% v/v. This trend correlated with what is understood from the literature of the role of detergent in VLP liberation. Increasing the amount of detergent would result in more effective disassociation of the HBsAg particle from the ER (Chi *et al.*, 1994) and promote dispersion of membrane material (Sitrin and Kubek, 1992) thereby increasing the surface area for disassociation of HBsAg to occur and, at a molecular level, facilitate inward budding of the VLP from the ER membrane (Smith *et al.*, 2002; Kreibich *et al.*, 1982).

However, when Triton X-100 concentrations were increased above 0.5% v/v, the HBsAg activity levels appeared to decrease. Since the ELISA technique correlates to

HBsAg activity, the decrease in recovery level observed is most likely to be a reflection of a decline in HBsAg antigenicity. The loss of activity observed is consistent with the findings of Gavilanes *et al.* (1990) and is attributed to HBsAg delipidation. Excess Triton X-100 present when its concentration is increased above a specific threshold could react with and remove lipid constituents from the HBsAg particle. Since the lipid moiety plays a critical role in maintaining the structural properties of the HBsAg, delipidation would lead to conformational changes which are antigenically unfavourable. [The HBsAg delipidation study to provide further evidence for this is discussed in the following section.]

In a separate study, to investigate if such a loss of antigenic activity were reversible, samples after the detergent step were diluted such that the resulting detergent concentration was equal to 0.1% v/v Triton X-100. If indeed the loss of antigenic activity due to the action of the detergent were reversible, the overall product yield estimated would be expected to increase when the detergent content in the sample were diluted down. A mass balance was carried out on the estimated HBsAg concentration before and after dilution. The results did not show any improvement in HBsAg yield after dilution, suggesting that the detrimental effect of the detergent on HBsAg is most likely irreversible.

The results in **Figure 4.3** indicate an apparent increase in HBsAg recovery at Triton X-100 concentrations above 2% v/v. It is likely that the species formed at Triton X-100 concentrations above 2% v/v are non-native VLP particles. As previously described, a mature HBsAg particle is assembled from ~ 100 copies of the 24 kDa monomeric proteins (p24s) (Peterson, 1987). Following complete delipidation it is likely that the p24s monomeric proteins form thermodynamically stable micelles in excess Triton X-100. [Triton X-100 has a low critical micelle concentration of 0.013%

v/v Triton (Sigma Product Information Sheet).] This species, as a result of its antigen orientation could show improved antigenicity as has been previously reported (Howard er al, 1982; Sanchez *et al.*, 1983; Skelly *et al.*, 1981).

Further studies to investigate the formation of antigenic polypeptide micelles and also the possibility of controlled disassembly and reconstitution of HBsAg to improve the homogeneity of the HBsAg particles may be warranted. However, these are beyond the scope of this study. In this study, the recovery of native HBsAg is of interest and the results clearly indicate that there is an optimal range of detergent concentrations necessary to ensure high levels of recovery and without compromising particle antigenicity.

4.3.3 Further investigation into HBsAg delipidation

HBsAg delipidation due to the effect of high Triton X-100 concentrations (>0.5% v/v) was further investigated. The motive of this study was to detect constituent lipid components which may have been removed from the native HBsAg due to the action of the detergent. The HPLC technique employed for this analysis detects lipids primarily on the basis of their functional groups.

In this study, cell homogenate was initially treated at a low Triton X-100 concentration of 0.1% v/v to ensure recovery of native HBsAg particles. Centrifugation was carried out following HBsAg liberation for the removal of membrane material. XAD-4, a polyaromatic absorbent specifically for small hydrophobic compounds (Sigma product information sheet), was employed for the removal of Triton X-100 detergent. The efficiency of the XAD-4 step is shown in **Figure 4.4(a)**. The subsequent chloroform-methanol precipitation step removed protein components prior to HPLC analysis. Both the centrifugation and XAD-4 steps were important to prevent any

further contribution of host lipid into the process stream and the potential interference of Triton X-100 with the lipid analysis. The product stream from this step was denoted as Sample I. The chloroform-methanol precipitation step preceding HPLC analysis removed protein components so that only soluble lipids were analysed. Lipid components in Sample I that were detected are shown in **Figure 4.4(b).** Based on the previous HBsAg activity analysis, no HBsAg delipidation was expected at 0.1% v/v Triton X-100. Hence, peaks A, B and D would represent host lipid components released into the process stream.

Sample I was divided into three aliquots. The first aliquot was kept at 0.1% v/v Triton X-100 as the control. The second and third aliquots were treated with Triton X-100 stock solution to mimic medium (0.7% v/v) and high (7% v/v) Triton X-100 concentrations and these were denoted as samples II and III, respectively. Since cell membrane material had been removed by centrifugation, excess Triton X-100 present in samples II and III could not lead to further release of lipid contaminants from the host membrane. However, if the HBsAg particles were susceptible to delipidation, excess Triton X-100 present in samples II and III could release the lipid constituent from the native HBsAg into the process stream. As previously, following a period of incubation, an XAD-4 step was carried out for removal of Triton X-100.

HPLC lipid profiles for samples II and III are shown in **Figures 4.4(c)** and **(d).** Peaks A, B and D observed from samples II and III were similar to those from sample I which was as expected since all three samples were originally from the same VLP liberation step using 0.1% v/v Triton X-100. However, at a retention time of 14 minutes, a baseline shift was observed for the medium Triton X-100 concentration mimic (sample II) and a distinct peak, Peak C, was present only for the high Triton X-100 concentration mimic (sample III). Peak C was not an artifact from Triton X-100 as

the retention times are different. In fact, the retention time of peak C suggested that the species involved is a phospholipid. [The presence of phospholipids and the role of phospholipids in HBsAg has been reported (Vanlandschoot *et al.*, 2003).] Furthermore, the source of this lipid could not be a contaminant from host cell membrane material since, as noted earlier, this had been effectively removed by centrifugation. Pure native VLP which comprises proteins and lipids when precipitated by chloroform-methanol would yield a lipid-free material as shown in **Figure 4.4(a)**. It follows therefore that in this case of HBsAg material, liberation of lipid from the antigen itself must be the source of lipid peak C seen in **Figure 4.4(d).** [Repeats of the delipidation study with purified HBsAg may have proven helpful to provide further evidence for this delipidation phenomenon although that was not performed here due to the lack of material availability.]

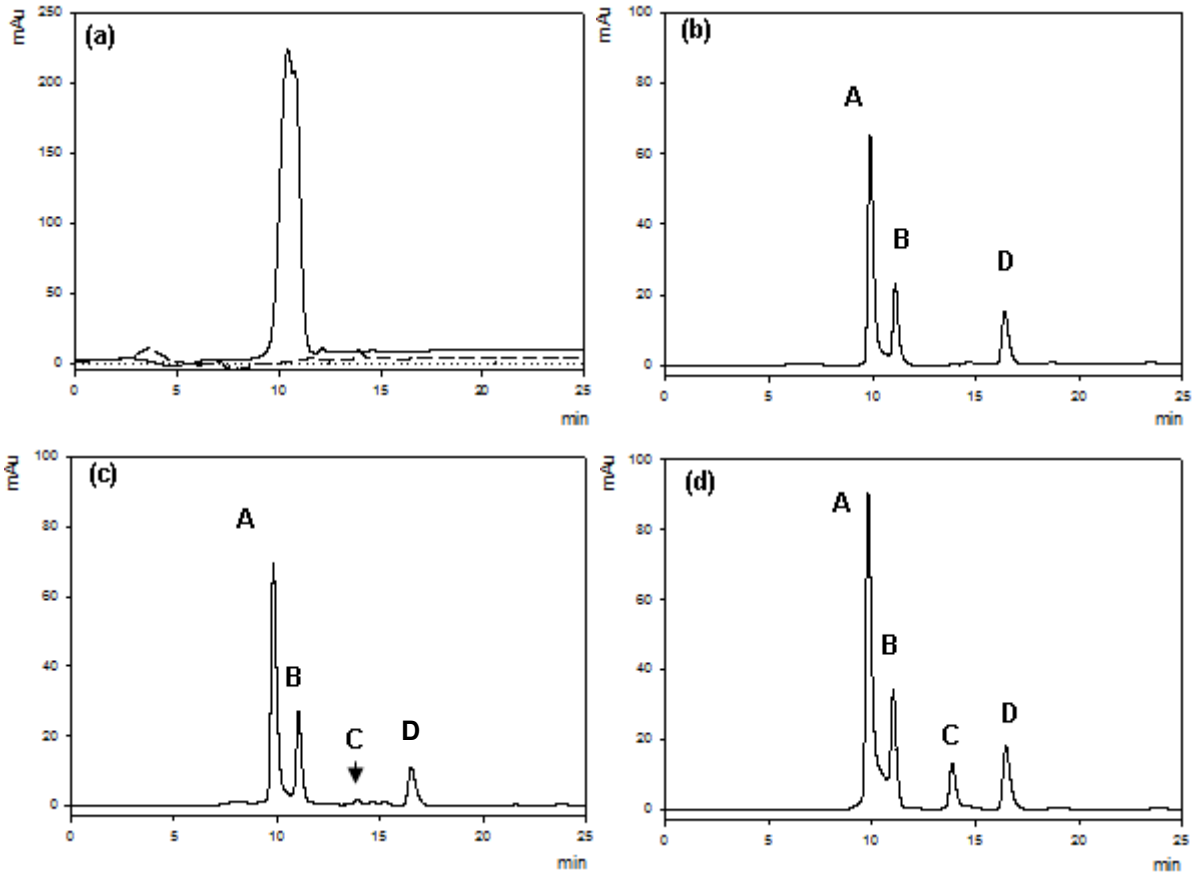


***Figure 4.4**: Chromatogram **(a)** shows the efficiency of Triton X-100 removal by XAD-4. (——) and (- - - -) represent the profiles for 7% (v/v) Triton X-100 in phosphate buffer before and after the XAD-4 step, respectively. (·····) was obtained when pure native HBsAg was analysed. Chromatogram **(b)** shows the lipid profile for the process stream recovered using 0.1% v/v Triton X-100 (Sample I). Chromatograms **(c)** and **(d)** were obtained when the process stream was spiked with Triton X-100 stock solution to mimic 0.7% v/v and 7% v/v Triton X-100 (Samples II and III). Peaks A, B and D correspond to host lipid contaminants and are similar for all samples. Peak C is only observed at high Triton X-100 concentrations and is attributed to HBsAg delipidation.*

4.3.4 Exploring the role of Triton X-100 in maintaining HBsAg liberation

Understanding the role of detergent in maintaining HBsAg liberation is crucial if potential product loss due to re-association of HBsAg to membrane components is to be minimised. This issue is likely to be most significant during the centrifugation step for removal of cell debris. Sedimentation of cell debris and the forces involved are likely to enhance the entrapment of HBsAg in the cell pellet. In this study the role of Triton X-100 in preventing HBsAg product loss during centrifugation was investigated.

In a typical primary recovery process, as shown in **Figure 4.1**, Triton X-100 is present during the centrifugation step for clarification of the stream from membrane debris. To investigate the role of Triton X-100 during this step, the process sequence shown in **Figure 4.1** was altered such that the XAD-4 step was carried out to remove Triton X-100 prior to the recovery of HBsAg product from cellular components by centrifugation. The level of HBsAg in the supernatant after centrifugation was compared to the level of product recovery at the end of primary recovery if the conventional route shown in **Figure 4.1** were adopted. From **Figure 4.5** it is evident that the recovery of HBsAg was lower when centrifugation was carried out in the absence of Triton X-100. The result suggests that the Triton X-100 played an important role during centrifugation in preventing re-association of HBsAg to the membrane components. Thus in terms of process design it is vital to ensure that the removal of cellular material and contaminating solids precedes the XAD-4 step.

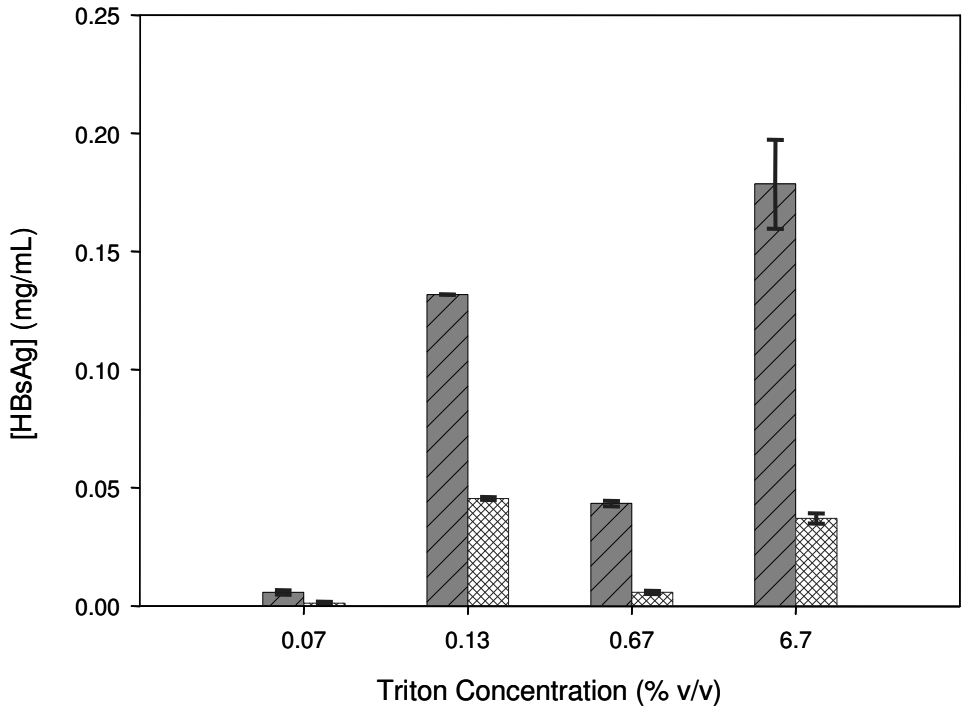


Figure 4.5*: Comparison of recovery level of HBsAg when Triton X-100 was absent (▨) during the centrifugation and when Triton X-100 was present (▨). Error bars correspond to standard deviation from triplicate samples.*

4.3.5 Investigating the effect of Triton X-100 on particle size characteristics

From this delipidation study and findings reported by others (Smith *et al.*, 2002; Kreibich *et al.*, 1982), it is likely that the detergent employed impacts recovery at a molecular level, specifically the degree of HBsAg release from the ER. Particle size analysis using dynamic light scattering (DLS) was carried out to probe further the role of Triton X-100 in this. The samples after primary recovery were highly impure. The challenges associated with the use of DLS in the presence of large contaminating particles have been discussed elsewhere (Holwill *et al.*, 1995; Madani *et al.*, 1991; Tsoka *et al.*, 1999). To prevent potential interference from larger-sized contaminants,

such as residual cell debris and lipid aggregates, a degree of size-based separation prior to DLS analysis was necessary.

In this study, the size of HBsAg populations recovered using 0.1% v/v, 0.4% v/v and 0.7% v/v Triton X-100 were investigated. Separation was carried out using size exclusion chromatography (SEC) and the fractions containing HBsAg were identified by an ELISA assay. These fractions were filtered using a 0.22 µm membrane filter prior to DLS analysis. Triton X-100 in the process stream could act as a surfactant minimising protein aggregation but in this study this was expected to be minimal since excess TritonX-100 would have been removed by the XAD-step.

DLS analysis for early antigenically active fractions from SEC showed no significant difference in particle size distribution. Native HBsAg particles were expected to be within the 20-30nm size range based on the diameter of HBsAg reported in literature (Zhou *et al.*, 2006). The early fractions for all samples showed mean values of approximately 100nm. This is likely to be caused by HBsAg aggregation which may have been aggravated by the SEC separation step. However, for later antigenically active fractions, differences in particle size distribution between the samples were observed. **Figure 4.6** shows that higher mean values were obtained for the low Triton X-100 sample (0.1% v/v Triton X-100). This result suggests that at low Triton X-100 concentrations, residual ER material may still be attached to the HBsAg particles, making them appear large. Furthermore, this species may also be more prone to aggregation owing to the hydrophobicity of the residual ER. These ideas concur with earlier hypotheses (Smith *et al.*, 2002; Kreibich *et al.*, 1982) that detergent may have a role in promoting inward budding of the HBsAg from the ER. Residual ER obstructing the antibody binding epitopes on the HBsAg may also explain the low antigenicity

measurements at 0.1% v/v Triton X-100. In order to verify these hypotheses, electron microscopy studies may be useful.

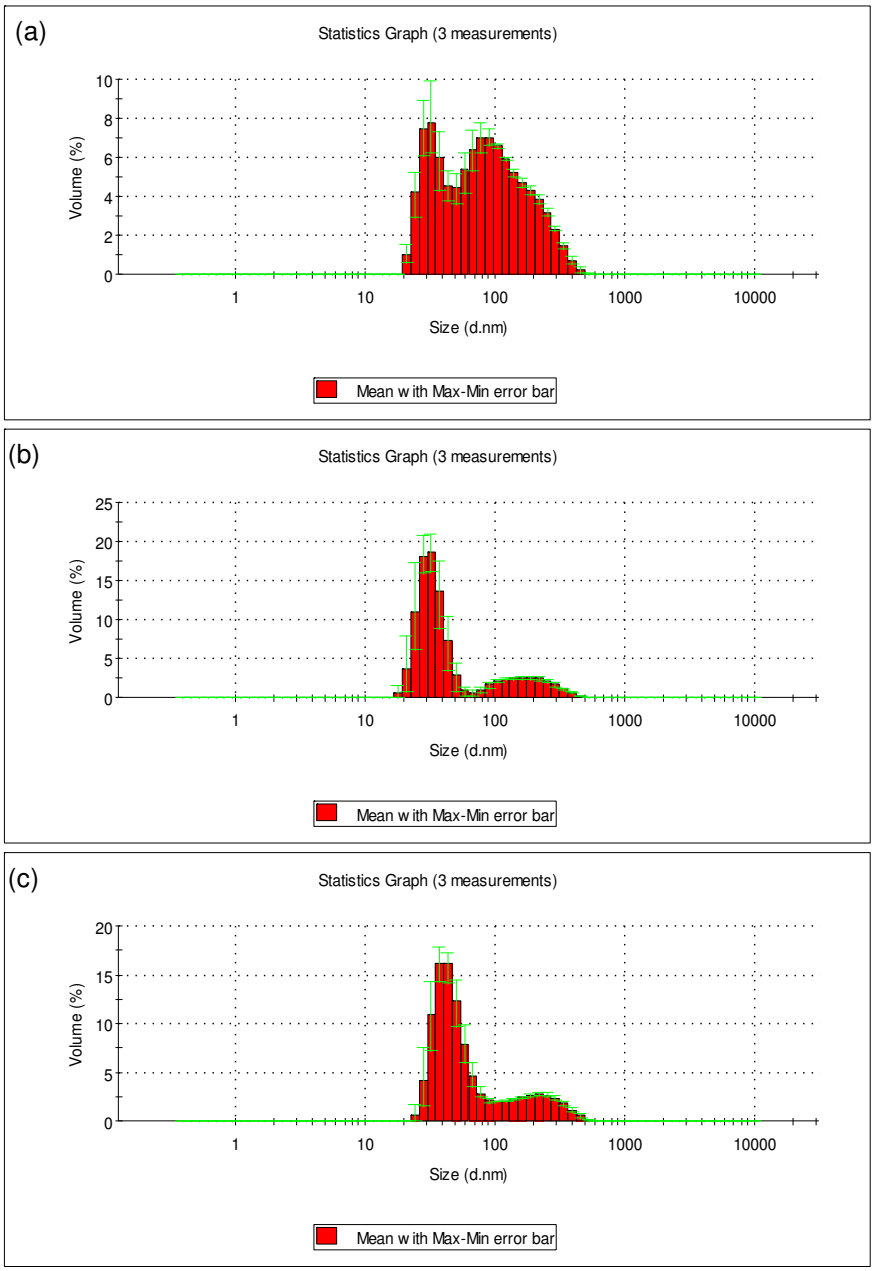


***Figure 4.6**: Comparison of particle size distribution (PSD) obtained using DLS analysis. Histograms (a), (b) and (c) correspond to PSDs of samples recovered using 0.1% v/v, 0.4% v/v and 0.7% v/v Triton X-100 respectively. The PSDs above were for later active fractions from SEC. The earlier active fractions from SEC gave mean particle size of approximately 100nm and no statistically significant differences were observed between the different Triton X-100 samples.*

4.3.6 Determination of the impact of Triton X-100 on characteristics of the process stream

The effects of the Triton X-100 conditions employed during the liberation step on the levels of host protein and lipid contaminants were investigated. The yeast ER, as well as other membranous organelles, is rich in lipid and contains various membrane proteins for cell metabolism. During the detergent step, recovery of HBsAg is likely to be accompanied by release of host lipids and proteins. Increasing the Triton X-100 concentrations employed in this step could result in higher levels of contamination. Characterisation of the process stream is therefore important since the level of contamination could affect the performance of subsequent downstream operations.

The lipid content in the process streams at the end of primary recovery under different Triton X-100 concentrations was determined using HPLC lipid analysis. Inclusion of the XAD-4 step in primary recovery process was important for the removal of excess Triton X-100 to prevent its interference with the analyses. In **Figure 4.7**, chromatograms **(a)** and **(b)** correspond to the lipid profile when 7% v/v and 0.1% v/v Triton X-100 were used respectively. The retention time for peak X is similar to that for ergosterol which has been observed in wild type *S. cerevisiae* strains. Peak Z is likely to be a phospholipid component while peak Y, which was observed at high Triton X-100 concentrations was attributed to delipidation of HBsAg as discussed previously. The total peak area determined from HPLC was plotted against the different concentrations of Triton X-100 employed (**Figure 4.8**). The results clearly suggest that the level of lipid contamination is significantly influenced by the Triton X-100 concentration.

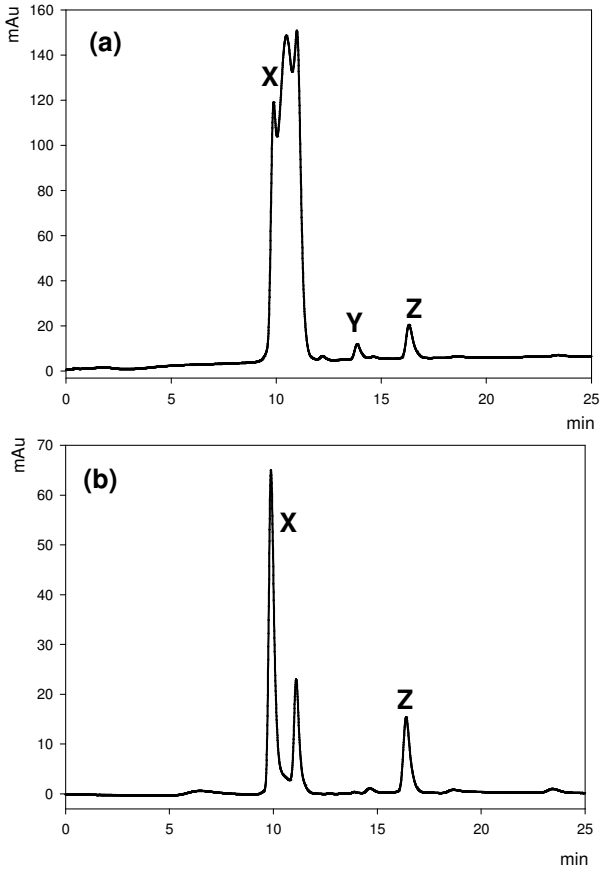


***Figure 4.7**: Chromatograms **(a)** and **(b)** show lipid profiles at the end of primary recovery when 7% v/v and 0.1% v/v Triton X-100, respectively, were used for the HBsAg liberation step.*

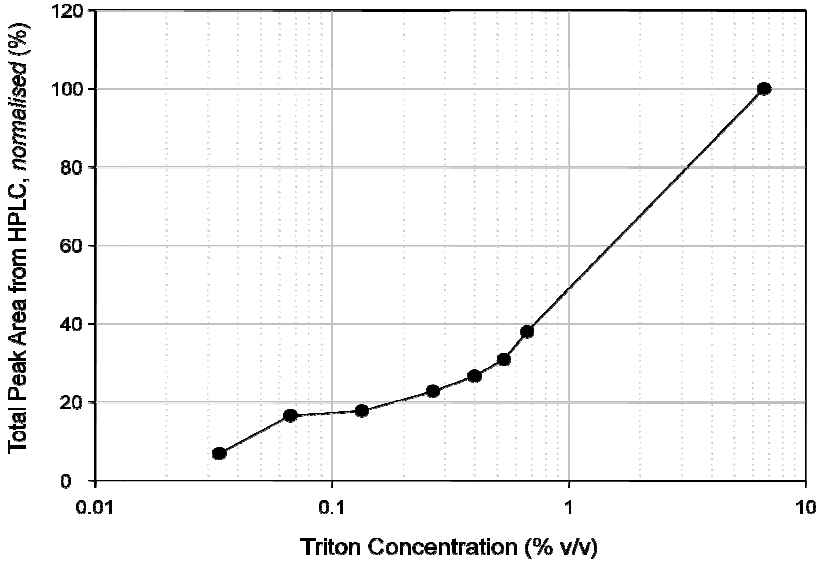


***Figure 4.8**: The total peak area from HPLC lipid analysis under different Triton X-100 concentrations employed during the HBsAg liberation step. Y-axis values have been normalised by denoting total peak area for 7% v/v Triton X-100 as 100%.*

The process streams following primary recovery were also analysed in terms of total protein. Total protein concentration was higher when more concentrated Triton X-100 solutions were employed. In **Figure 9**, protein purity is expressed as the ratio of HBsAg to total protein. Initially an apparent increase in HBsAg purity was observed with increasing Triton X-100 concentration and this is most likely due to improved recovery of HBsAg. However at Triton X-100 concentrations above 0.2% v/v, a decrease in the level of HBsAg purity was observed. This is due to the combined effect of the increase in host protein contamination and the decrease in antigenic HBsAg at high Triton X-100 concentrations. [Attempts were also made to analyse HBsAg purity using SDS-PAGE. This however proved unsuccessful due to the poor resolution of the bands obtained which was a result of the crude nature of the cell lysate samples.]

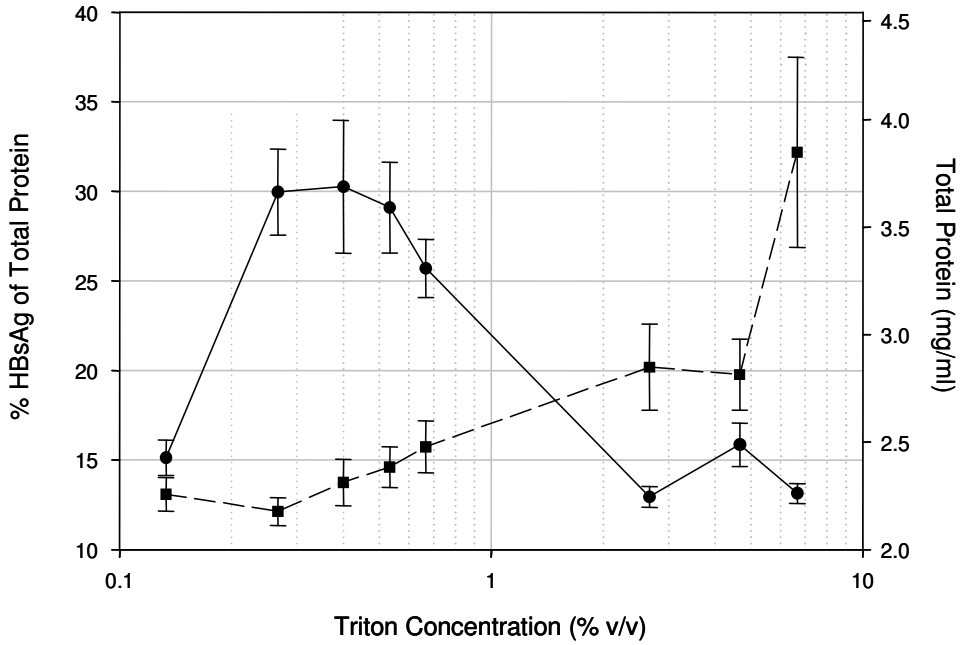


***Figure 4.9**: Recovery of HBsAg as a percentage of total protein under different Triton X-100 concentrations (—●—) is plotted on the primary axis. The proportion of HBsAg reflects protein purity. The secondary axis shows total protein concentration at the end of primary recovery (— ■ —) with increasing Triton X-100 concentrations. Error bars correspond to standard deviation from triplicate samples.*

The combined protein and lipid results indicate that higher levels of contamination resulting from the use of excess Triton X-100 could raise specific challenges for subsequent downstream operations. Examples might include fouling of membrane filtration (Maartens *et al.*, 1998) and chromatographic stages (Pampel *et al.*, 2007) due to high levels of lipid in the process stream. The presence of contaminants in the process stream could also affect the purification performance achieved during downstream operations.

4.3.7 Investigating the indirect impact of the detergent step on UF performance

As demonstrated, the amount of host lipid and contaminating proteins present in the process streams could vary depending on the amount of Triton X-100 used in the VLP liberation step. With reference to **Figure 4.1**, the process stream after primary recovery is normally subjected to an ultrafiltration (UF) step to achieve the appropriate buffer conditions required for chromatography. Thus, the sensitivity of UF performance to processing streams resulting from the use of different concentrations of Triton X-100 was investigated. The study was carried in an ultra-scale down (USD) device to mimic actual processing conditions. The relationship describing a membrane filtration process derived from Darcy's law is shown in **Equation 4.1** below.

$$\frac{1}{A}\frac{dV}{dt} = \frac{\Delta P}{\mu R_m} \qquad \text{(Equation 4.1)}$$

where A is the effective membrane area (m^2), V the permeate volume (m^3), t filtration time (s), P the transmembrane pressure drop (N m^{-2}), μ dynamic viscosity and R_m membrane resistance (m^{-1}). The term $\frac{1}{A}\frac{dV}{dt}$ represents flux. Flux and transmembrane pressure (ΔP) are important parameters.

Flux versus TMP curves for process streams originally generated using 0.1% v/v, 0.4% v/v and 0.7% v/v Triton X-100 are shown in **Figure 4.10**. The samples investigated were obtained at the end of primary recovery where Triton X-100 had been removed from the process stream by the application of the XAD-4 step. This was important to ensure that the filtration performance was not influenced by the presence of the detergent.

No significant difference in critical flux was observed for all three Triton X-100 concentrations but the transmembrane pressure required to achieve the critical flux was lowest for the process material prepared using 0.1% v/v Triton X-100. In order to understand better the possible reasons for these observations, the direct impact of host contaminants on membrane resistance during filtration was investigated. **Equation 4.1** can be rearranged to give **Equation 4.2** shown below.

$$\frac{\Delta P}{[\frac{1}{A}\frac{dV}{dt}]} = \mu R_m \qquad \text{(Equation 4.2)}$$

The flux versus TMP curves in **Figure 4.10** show very linear correlations ($R^2 >$ 0.98) below the critical flux points. The left hand term of **Equation 4.2** is the reciprocal of the gradient of the flux versus TMP curve. Determination of the viscosity of the samples showed that each of these to be close to the theoretical value for water, 0.0091 $kgm^{-1}s^{-1}$ (T = 297K) indicating that impact of contaminants on viscosity was small. By

application of **Equation 4.2**, the membrane resistance (R_m) for the different samples was calculated and a summary of the results is provided in **Table 4.1**.

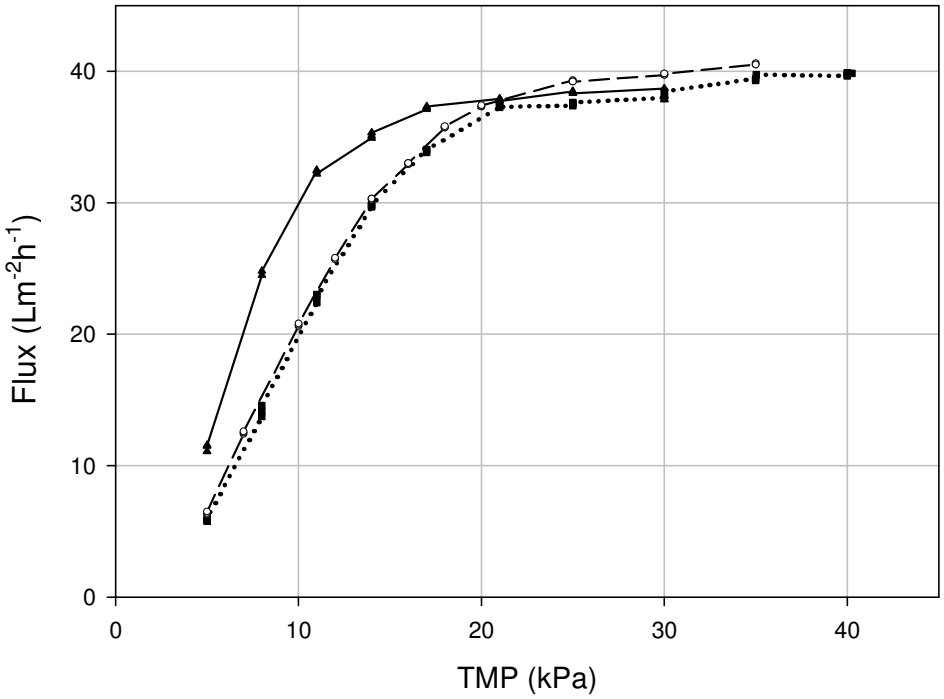


***Figure 4.10**: Comparing flux versus TMP curves for process streams recovered using different concentrations of Triton X-100: 0.1% v/v Triton X-100 (——▲——), 0.4% v/v Triton X-100 (····■····) and 0.7% v/v Triton X-100 (- - ○ - -).*

***Table 4.1**: Comparing viscosity and membrane resistance for samples recovered at the end of primary recovery under different Triton X-100 concentrations. All experiments were carried out at 297 K.*

Triton X-100 concentration [% v/v]	**TMP / Flux [x10^7 Pa / ms^{-1}]**	**Viscosity [x10^{-3} Pa s]**	**Membrane resistance [x10^{10} m^{-1}]**
0.1	1.69	0.96	1.76
0.4	2.47	1.03	2.39
0.7	2.57	1.04	2.48

As expected, R_m is influenced by the Triton X-100 concentration employed in the liberation step. Higher membrane resistances for samples recovered using 0.4% v/v and 0.7% v/v Triton X-100 reflect more severe membrane fouling and were consistent with the earlier lipid contamination analysis (**Figure 4.8**). The levels of contaminating protein also differed across this range of Triton X-100 (**Figure 4.9**) but vary to a much lower extent than did the lipid content which was probably therefore the major source of the membrane fouling. It is not clear why the level of increase in R_m was small from 0.4% to 0.7% Triton X-100. This may be due to the nature of the fouling or cake formation that occurred on the membrane surface at these concentrations. Such analysis was however considered to be beyond the scope of this study.

Lower membrane resistance was encountered when HBsAg liberation was carried out using lower Triton X-100 concentrations. Based on filtration performance alone, low Triton X-100 conditions are thus favoured. The reduced extent of membrane fouling as a result of minimal release of host lipid would allow the use of lower pressure operations and could result in an improvement in membrane lifespan. More importantly, the potentially gentler operation could also promote HBsAg stability and reduce problems of shear-induced protein denaturation and aggregation.

4.4 Conclusions

The results obtained demonstrated that the use of Triton X-100 is superior for the liberation of HBsAg from the ER and its operating concentration has significant impact on the effectiveness of the HBsAg recovery process. Operating below the range of effective concentrations of Triton X-100 was found to lead to incomplete HBsAg liberation and poorer antigenic activity. Excess detergent, on the other hand, was shown

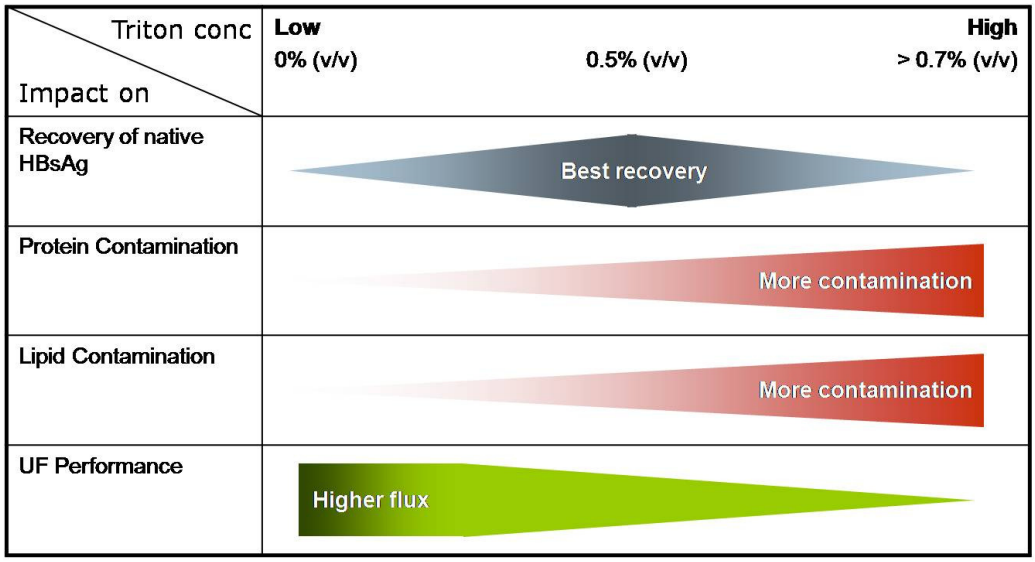
to cause HBsAg delipidation which also resulted in reduced particle antigenicity. From the perspective of process performance there is no obvious advantage in the use of high Triton X-100 concentrations for the process. Increasing the amount of Triton X-100 employed would raise the level of host protein and lipid contamination which in turn would have a negative impact on the performance of downstream operations. The results obtained in this study are summarised in **Table 4.2**.

Based on the system investigated, the most suitable range of Triton X-100 concentrations to ensure good recovery of HBsAg and maintenance of its antigenic properties is likely to be between 0.2% v/v and 0.5% v/v. Triton X-100 concentrations below 0.2% v/v showed poor yields of HBsAg. Based on the particle size analysis, it is likely that residual ER material remain associated with the HBsAg particle under such conditions. This could hinder the immunogenic recognition of the antigen thereby reducing the efficiency of the vaccine material generated. Reduced HBsAg activity was also observed at Triton X-100 concentrations of above 0.5% v/v and this is attributed to HBsAg delipidation as previously discussed. The raised levels of host derived contaminants in addition to the loss of antigenic activity at such high detergent concentrations are unfavourable from the perspective of process efficiency. Choosing a specific concentration within the 0.2% v/v – 0.5% v/v range would depend on further trade-off considerations between product recovery and the level of contamination that can be tolerated by downstream operations. As a general guideline, product yield is typically given precedence over purification for such an early stage recovery operation thus operating close to the 0.5% v/v upper limit would be ideal.

In conclusion, the issues presented in this study could be valuable as a guide to the process design of future generation of lipid-based VLP vaccine candidates. Although the type of detergent and the specific range of optimal operating

concentrations may differ from system to system, the framework and insights gained from this study could be employed as a platform for process selection and optimisation.

***Table 4.2**: Schematical representation of the effect of detergent conditions on various process parameters*



Having established the trade-off conditions for the detergent-mediated HBsAg liberation step to ensure maximal HBsAg recovery with minimal co-extraction of host-derived contaminants, subsequent studies were performed to improve further the purity of the processing stream entering later stage chromatographic operations. The use of precipitation methods and a strategy for selective product recovery for this purpose are illustrated in the following chapters.

Chapter 5

HBsAg precipitation with polyethylene glycol (PEG) and ammonium sulphate

5.1 Introduction

The purification of HBsAg expressed in yeast requires a complex and multistage process to deal with the high levels of host cell lipid and protein contaminants present. Release of such contaminants into the process stream is exacerbated by the use of detergents such as Triton X-100 for the liberation of HBsAg from the endoplasmic reticulum (ER) (Kee *et al.*, 2008). The presence of these contaminants could affect the performance of downstream operations (Kee *et al.*, 2008; Pampel *et al.*, 2006; Bracewell *et al.*, 2008). Furthermore, the exposure of the HBsAg product to protease contaminants could lead to product degradation (Chi *et al.*, 1994). Hence the removal of such contaminants early in the process is favourable.

Currently, the manufacturing process for HBsAg relies heavily on the use of chromatographic operations (Koen *et al.*, 2005; Sitrin and Kubek, 1992, Dekleva, 1999) which offer high levels of resolution but are expensive and have low-throughputs (Przybycien *et al.*, 2005). The ability to reduce the burden and reliance on chromatography is attractive (Leser and Asenjo, 1992; Tsoka *et al.*, 2000, Przybycien *et al.*, 2005) and one of the means of achieving this is by introducing an additional separation step to achieve partial purification prior to downstream processing.

Of the various methodologies available, selective protein fractionation by precipitation remains popular as it affords both purification and concentration in a single step. Polyethylene glycol (PEG) is a commonly employed class of precipitatant particularly for the separation of virus-like particles because of its selectivity for larger size proteins. Other benefits of PEG include its low potential for denaturation of proteins (Polson *et al.*, 1964) and its inert nature (Ingham *et al.*, 1978). In fact the first generation hepatitis B vaccines derived from the plasma of hepatitis B carriers relied on PEG precipitation and centrifugation techniques (Einarsson, 1983). Low concentrations of PEG (typically below 5% w/v) employed for recovery of HBsAg from human plasma have been reported by other groups (DeRizzo *et al.*, 1972; Vnek and Prince, 1976). In such cases to achieve the level of purity required, multiple fractionation "cuts" had to be carried out.

The use of salts, such as ammonium sulphate, as a fractionation agent for HBsAg from blood plasma has also been demonstrated (Peterson *et al.*, 1981). In general, salts lack selectivity and for the same level of precipitation, larger quantities of the precipitating agent of up to 40-50% w/v may be required for protein precipitation (Van Wijnendaele *et al.*, 1987). This could potentially lead to waste disposal problems. An inverse strategy could be adopted in which the contaminants are precipitated out at lower salt concentrations leaving the protein product in solution. The benefit is that the protein product undergoes no phase change and the challenges of product recovery by precipitate resolubilisation can be avoided. An example of this has been demonstrated in the reduction of lipid contaminants prior to a hydrophobic interaction chromatography step (Bracewell *et al.*, 2008).

Although the use of PEG and ammonium sulphate have been successful in the purification of human plasma HBsAg, it may not be necessarily so for recombinantly

expressed HBsAg particles which are not completely identical in form. In yeast, there is limited cross-linking of the recombinant HBsAg during its production. Rather it is during the purification process and KSCN treatment post-purification that the disulphide bond formation of the mature particle is achieved (Zhao *et al.*, 2006; Wampler *et al.*, 1985). By contrast, HBsAg recovered from serum is already in the mature form.

This chapter investigates and compares the potentials of PEG and ammonium sulphate precipitation in the bioprocessing of recombinant HBsAg, specifically for contaminant reduction to reduce the burden on downstream chromatographic operations. PEG 6000 and PEG 8000 were chosen for this study as PEG with molecular weight within this range has previously been identified to be most suitable for VLP precipitation (Tsoka *et al.*, 2000). Similarly, different concentrations of ammonium sulphate solutions were evaluated. Key response factors were the level of product yield, the amount of precipitant required and the degree of purification afforded. From the perspective of process feasibility, the mechanism of separation for the precipitate from the mother liquor is equally important and the use of centrifugation and filtration were evaluated and compared in this light.

5.2 Materials and Methods

Crude cell lysate containing HBsAg for the precipitation studies were generated by fermentation (**Section 2.2**), homogenisation (**Section 2.3**) and detergent-mediated HBsAg liberation (**Section 2. 4**). The precipitation experiments using polyethylene

glycol (PEG) and ammonium sulphate were conducted as in **Section 2.5** and **Section 2.6**, respectively.

Sample analysis was performed using an ELISA-based assay (**Section 2.13.4**) for measuring the level of HBsAg present in the samples, a Bicinchoninic (BCA) assay for total protein determination (**Section 2.13.5**) and lipid analysis by HPLC (**Section 2.13.6**).

5.3 Results and Discussion

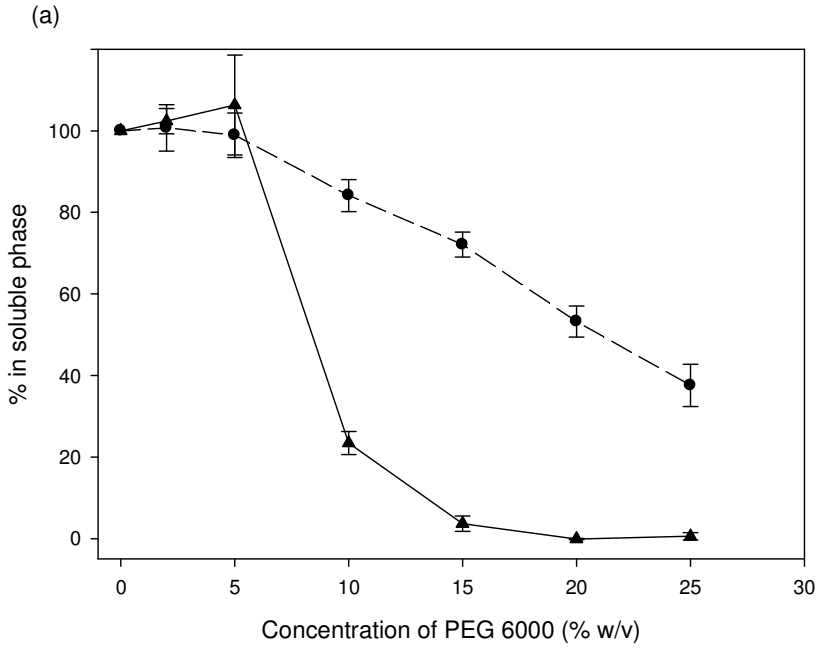
This study investigates the potential of including a precipitation step in the primary purification of recombinant HBsAg to achieve product enrichment and contamination reduction prior to high resolution chromatographic operations. Polyethylene gycol (PEG), a type of nonionic water soluble polymer and ammonium sulphate salt were chosen for this study as they represent two of the most common classes of precipitants employed for VLP particles. As mentioned in the introduction, product yield across the precipitation step and the level of product enrichment were used as the key parameters for evaluation.

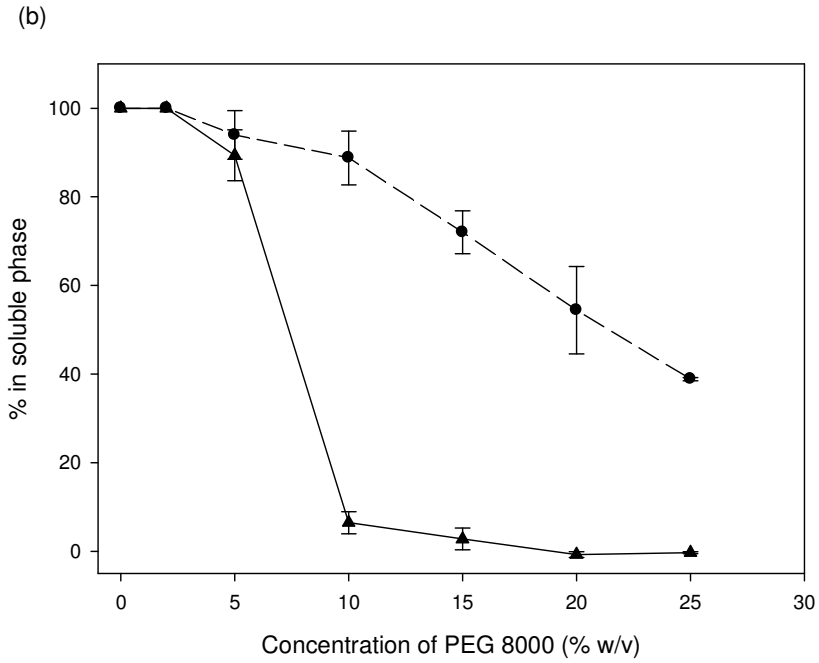
5.3.1 PEG precipitation chemistry and impact on purification and yield

PEG 6000 and PEG 8000 were evaluated in this study as these molecular weights have previously been identified to be most suitable for VLP fractionation (Tsoka *et al.*, 2000). Different concentrations of PEG were added to the cell lysate samples and following a period of agitation, the samples were centrifuged. The

concentration of the HBsAg product and that of total protein in the supernatant fractions were analysed by ELISA and BCA assay respectively. Based on the results, the solubility profiles of the HBsAg protein in comparison with total protein under different PEG concentrations were developed as shown in **Figure 5.1**. For PEG 8000 and PEG 6000, the HBsAg concentration in the soluble phase dropped below 10% at PEG concentrations above 10% w/v and 15% w/v respectively indicating successful precipitation of the HBsAg product under these conditions. Total protein concentrations in the soluble phase however were relatively high which means that whilst significant proportions of the HBsAg were precipitated, the total protein remained largely in solution. The greater sensitivity of the HBsAg particles, which are larger than the average proteins, to PEG precipitation is consistent with the findings of others (Juckes *et al.*, 1971; Tsoka *et al.*, 2000).

The fractionation diagram as shown in **Figure 5.2** allows comparison of the precipitation behaviour of PEG 6000 and PEG 8000. In the fractionation diagram, the percentage of HBsAg in solution was plotted against the percentage of total protein in solution. The diagonal line of zero fractionation represents conditions whereby no purification is achieved as the HBsAg and total protein are equally dispersed in both solids and soluble states. PEG 8000 appears to be the more effective agent as its curve is further displaced from the line of zero-fractionation. Highest HBsAg enrichment in the solids phase was achieved at 10% w/v PEG 8000 with an estimated product enrichment factor of ~ 8 fold. The highest product enrichment factor for PEG 6000 was achieved at a concentration of 15% w/v PEG and was ~ 5 fold.





Figures 5.1 (a) *and* ***(b)*** *show the solubility profile of the HBsAg product (—▲—) in comparison to the average protein (—●—) plotted against different concentrations of PEG 6000 and PEG 8000 during the precipitation step. Error bars indicate standard deviation from triplicate samples.*

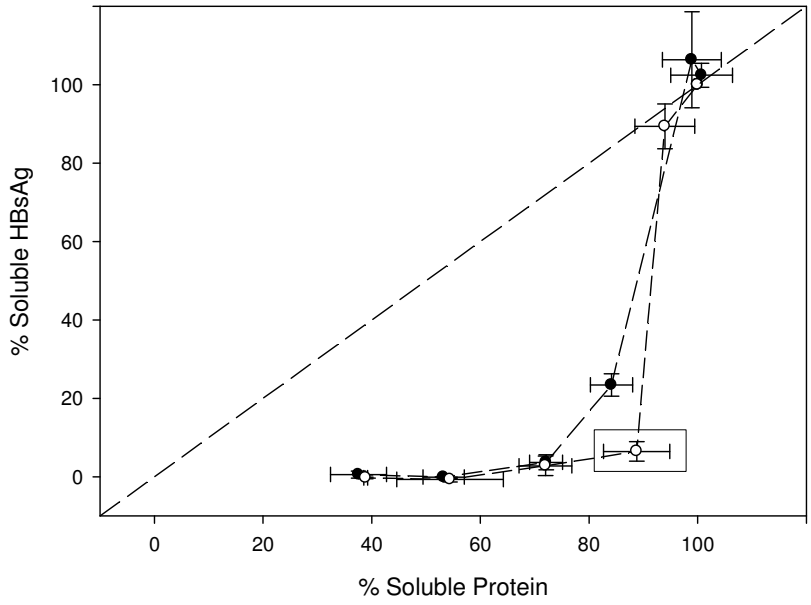


Figure 5.2: *The degree of HBsAg fractionation from total protein in the soluble phase for PEG 6000 (—●—) and PEG 8000 (—◇—) under different PEG concentrations. (- - - - -) is the line of zero fractionation at which the HBsAg and the average protein are equally partitioned in the solids and soluble phases. (▭) shows highest product enrichment. Error bars indicate standard deviation from triplicate samples.*

A lower amount of precipitant was required to achieve a given level of HBsAg precipitation for PEG 8000 in comparison to PEG 6000. For example, to achieve greater than 80% HBsAg precipitation, 10% w/v PEG 8000 was required in contrast to the 15% w/v required for PEG 6000. These findings are consistent with previous reports that the use of higher molecular weight PEGs yielded lower requirements of the precipitating agent (Tsoka *et al.*, 2000) and hence from a process design perspective are preferred.

In addition to the reduction in host protein contaminant levels, the use of PEG precipitation also showed potential for the removal of lipid contaminants. 10% w/v PEG

8000, which was identified as optimal for the removal of protein contaminants, also achieved an approximate 65% reduction in the level of host lipids (data not shown).

High levels of product enrichment however could occur at the expense of product yield. **Figure 5.3** was developed to investigate the relationship between the level of purification afforded and the maximum achievable yield assuming complete recovery and resolubilisation of the precipitate. PEG conditions achieving lower than 10% protein precipitation were not represented in this plot for the reason that their purification factors, calculated to be near infinity, were unrealistic. Based on **Figure 5.3**, it is evident that 10% w/v PEG which gives the highest purification factor of 8-fold and maximum theoretical yield of over 90% presents the best trade-off condition.

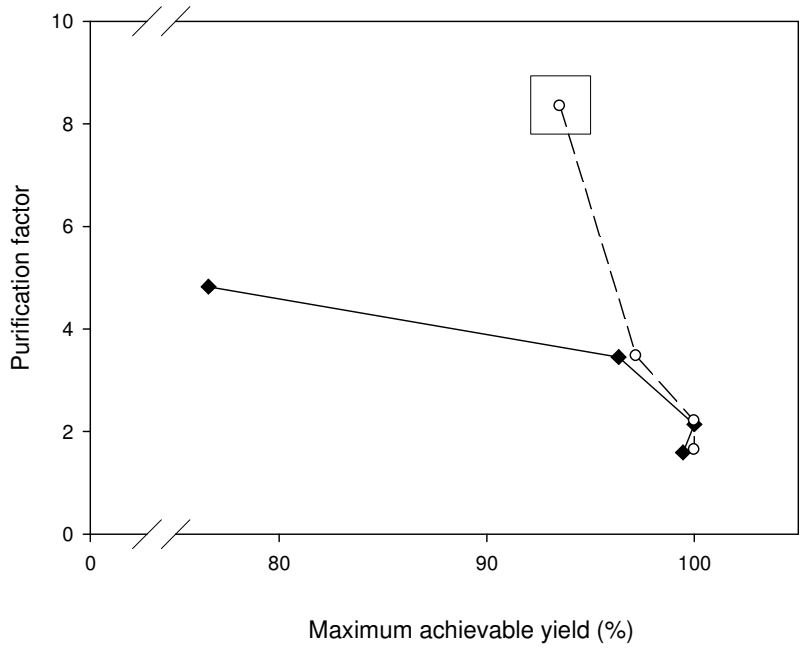


Figure 5.3: *Trade-off assessment of the degree of purification versus maximum achievable HBsAg yield for PEG 6000 (—◆—) and PEG 8000 (—◇—) at different PEG concentrations. (□) represents conditions for best trade-off.*

5.3.2 Precipitate recovery using centrifugation or filtration

Having established the purification potentials of PEG 6000 and PEG 8000, subsequent studies were carried out to evaluate the precipitation step yield. Other than the impact of the precipitating agent in causing a phase change, step yield also depends on the efficiency of the separation of the precipitates from the mother liquor and the resolubilisation of the recovered precipitate. The HBsAg yields achieved following precipitate recovery by centrifugation and resolubilisation are shown in **Figure 5.4**.

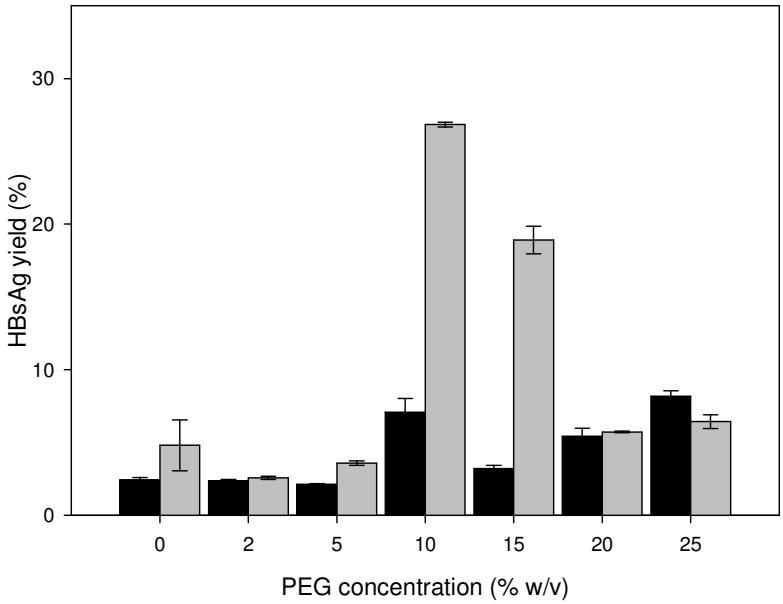


Figure 5.4*: HBsAg yield following resolubilisation of the precipitate recovered from the mother liquor using centrifugation for PEG 6000 ( ) and PEG 8000 ( ). HBsAg level of ~1 g corresponds to 100% recovery. Error bars indicate standard deviation from triplicate samples.*

On the whole, HBsAg product recovery was very low. The optimal range of PEG concentrations appears to be between 10% w/v and 15% w/v PEG with respect to the final product yield after the resolubilisation of the precipitate. At lower PEG

concentrations, there is insufficient PEG to precipitate the HBsAg and thus the HBsAg remains largely in solution. At higher PEG concentrations, the increased viscosity of the mother liquor impacts negatively the sedimentation characteristics of the HBsAg precipitates formed.

To circumvent the issue of low rates of precipitate sedimentation by centrifugation, the use of filtration as an alternative for precipitate recovery was investigated. As shown in **Figure 5.5**, significant improvement in HBsAg yield was observed using dead-end filtration. For PEG concentrations of 10% w/v and above, average HBsAg yields of 50% were achieved. Unlike in the case with centrifugation, no significant drop in HBsAg yield at PEG concentrations above 15% w/v was observed. This supports the hypothesis that the drop in yield observed for the centrifugation recovery option was primarily due to viscosity effects.

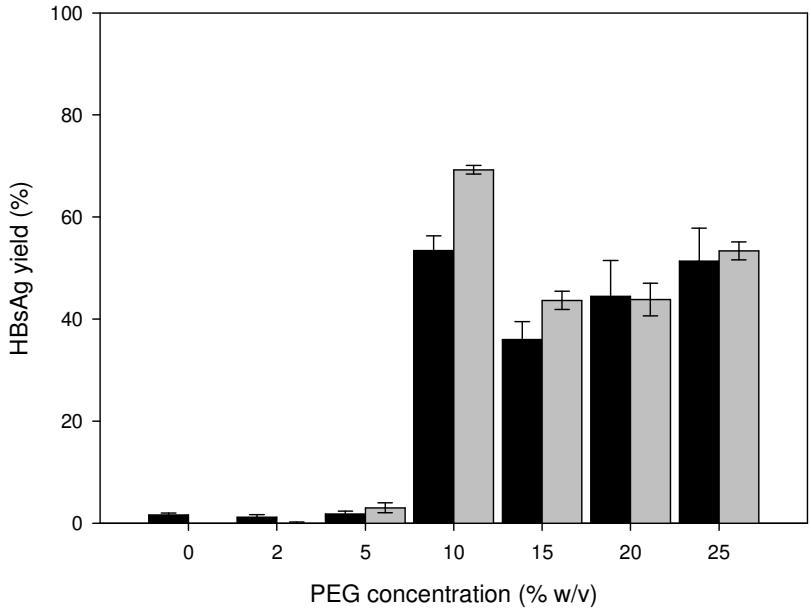


Figure 5.5*: HBsAg yield following resolubilisation of the precipitate recovered from the mother liquor using filtration for PEG 6000 (* ■ *) and PEG 8000 (* ▭ *). HBsAg level of ~0.2 g corresponds to 100% recovery. Error bars indicate the standard deviation from triplicate samples.*

Based on ELISA measurements, highest product yield of approximately 70% was measured at 10% w/v PEG 8000. Of the 30% product lost, an estimated 10% was found to be owing to unprecipitated HBsAg which remained in solution. The other 20% drop in ELISA activity measurement could be due to irreversible loss of antigenicity or incomplete resolubilisation of the precipitate. To investigate the latter, a buffer containing 0.2% v/v Triton was added to aid the resolubilisation process. However no improvement in the HBsAg yield was observed. Based on this, it is more likely that the primary cause for the drop in the HBsAg levels observed was the negative impact of precipitation on HBsAg antigenicity rather than the issue of poor resolubilisation.

In the experiment, the samples were compared against a filtered control. Hence, the effect of material loss due to irreversible adsorption on the filter membrane is not reflected in these calculations. A subsequent experiment was performed to quantify the magnitude of the filter losses and this was found to be ~ 20% which would reduce the maximum yield achieved using 10% w/v PEG 8000 to ~ 50%. The level of product loss on the filter is a function of the protein load per unit area of membrane. Typically high protein loading > 20 g/m^2 is recommended to minimise adsorption losses (Millipore Technical Publication). The low protein load experienced on the filter plate in this case, may have led to the overly pessimistic results and would represent the worst case scenario.

5.3.3 Comparison with historical precipitation data for human-derived HBsAg

Optimal precipitation conditions and step yields reported here for recombinant yeast HBsAg were found to be different from those reported in literature for the purification of HBsAg from human plasma. In the methodologies described previously (Vnek and Prince, 1976; DeRizzo *et al.*, 1972) multiple precipitation cuts using low concentrations (2 - 5% w/v) of PEG 6000 were employed with step yields of 80 - 90% HBsAg product. In our study however, using the same PEG precipitating agent, concentrations above 10% w/v were required and step yields obtained were lower at ≤ 70%. The difference in performance may be attributed to the physiochemical differences between recombinant HBsAg and the human-derived form. It has been reported (Wampler *et al.*, 1985; Zhao *et al.*, 2006) that limited cross-linking of the HBsAg occurs spontaneously both during production in yeast and ensuing downstream purification and that the maturation process relies on subsequent KSCN treatment and incubation at 37 ^{o}C. The human-derived HBsAg is known to be in the mature form whereas the recombinant HBsAg at this stage of the process would only have partial cross-linking. Owing to the extensive disulphide-bond formation and structural consolidation, mature HBsAg would have a more compact structure and reduced conformational flexibility (Zhao *et al.*, 2006). This may also explain why human-derived HBsAg are more amenable to PEG precipitation and more resistant to losses in antigenicity during the process.

5.3.4 Ammonium sulphate precipitation chemistry and impact on purification and yield

Ammonium sulphate is another widely employed precipitating agent. High concentrations in the region of 40% w/v are typically required to precipitate proteins out of solution (Van Wijnendaele *et al.*, 1987). However, the use of ammonium sulphate has also been demonstrated for the precipitation of lipids and this was found to occur at lower concentrations, ~20% w/v (Bracewell *et al.*, 2008). Precipitation using low concentrations of ammonium sulphate for lipid removal whilst maintaining the HBsAg in solution may represent an attractive alternative to circumvent the issues of poor HBsAg precipitate resolubilisation and activity losses noted earlier with PEG.

In this study cell lysate samples were treated with ammonium sulphate solutions to achieve final concentrations of 0 – 25% w/v. Following a period of incubation, the samples were centrifuged to promote lipid separation. It was observed that the lipid precipitates formed a waxy upper layer consistent with the earlier findings (Bracewell *et al.*, 2008). The supernatant samples were analysed in terms of the amount of lipid and HBsAg in the soluble phase. Decreased lipid solubility with increasing concentrations of ammonium sulphate is as shown in **Figure 5.6**. Above ammonium sulphate concentration of 15% w/v no HBsAg product was detected in the supernatant (**Figure 5.7**). The results suggest that under these conditions, the HBsAg becomes almost completely co-precipitated with the lipid contaminants.

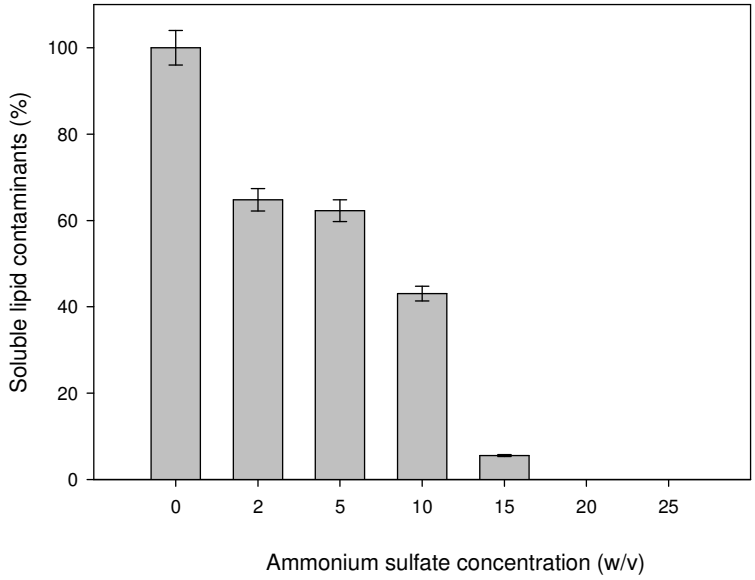


***Figure 5.6**: The level of lipid contaminants in the soluble phase following precipitation with different concentrations of ammonium sulphate. Error bars indicate standard deviation from triplicate samples.*

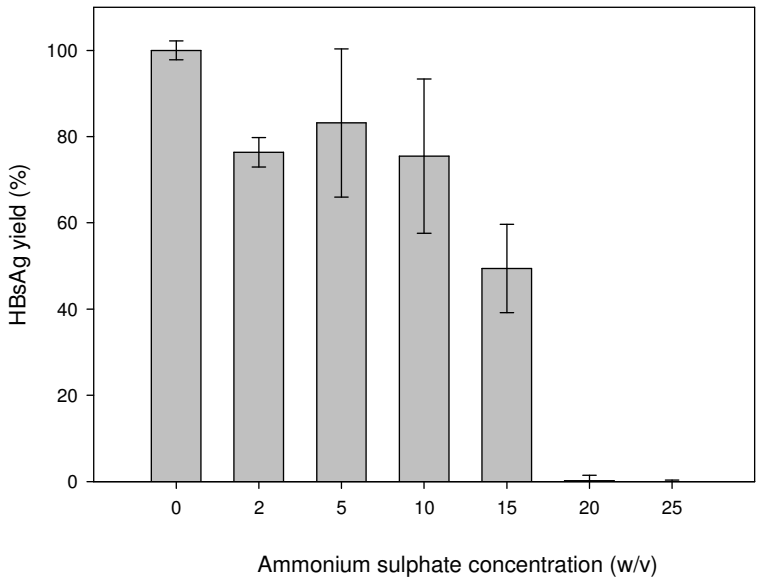


***Figure 5.7**: HBsAg yield following precipitation with different concentrations of ammonium sulphate solutions. HBsAg level of ~1 g corresponds to 100% recovery. Error bars indicate standard deviation from triplicate samples.*

To ascertain the separation efficiency of ammonium sulphate precipitation, a fractionation diagram analogous to that in **Figure 5.2** was generated as shown in **Figure 5.8**. Final ammonium sulphate concentration of ~ 15% w/v, indicated by "**X**" in the figure, is furthest from the line of zero fractionation and would give the highest degree of product enrichment. Under this condition, greater than 90% reduction of lipid contaminants was achieved with an HBsAg enrichment factor of ~ 8-fold.

Figure 5.9 which is analogous to **Figure 5.3** in the PEG precipitation study was developed to study the trade-off between the level of purification attained and product yield. The results show that the co-precipitation of HBsAg with the lipid contaminants could have a significant impact on product yield. The 8-fold lipid reduction achieved using 15% w/v ammonium sulphate was at the expense of product loss of up to ~ 50%.

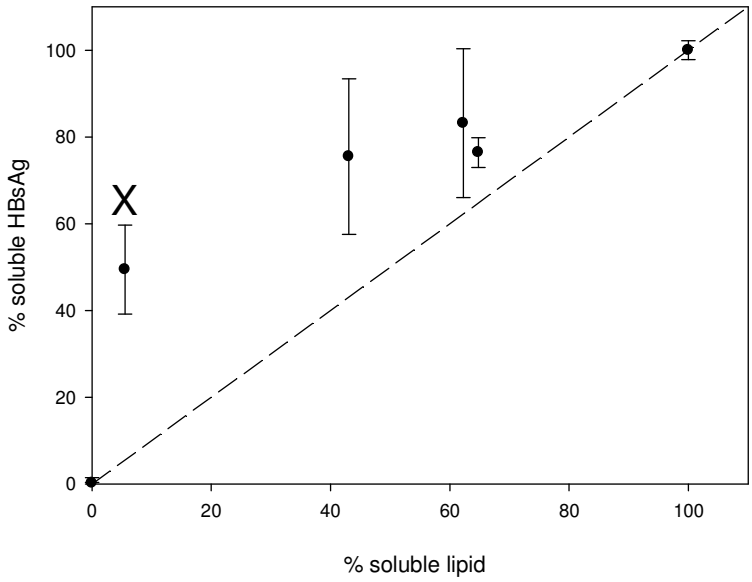


Figure 5.8: *The degree of HBsAg fractionation from host lipid using ammonium sulphate precipitation. (- - - - -) is the line of zero fractionation representing conditions at which the HBsAg and the host lipid contaminants are equally partitioned in the solids and soluble phases and (**X**) represents highest product enrichment. Error bars indicate standard deviation from triplicate samples.*

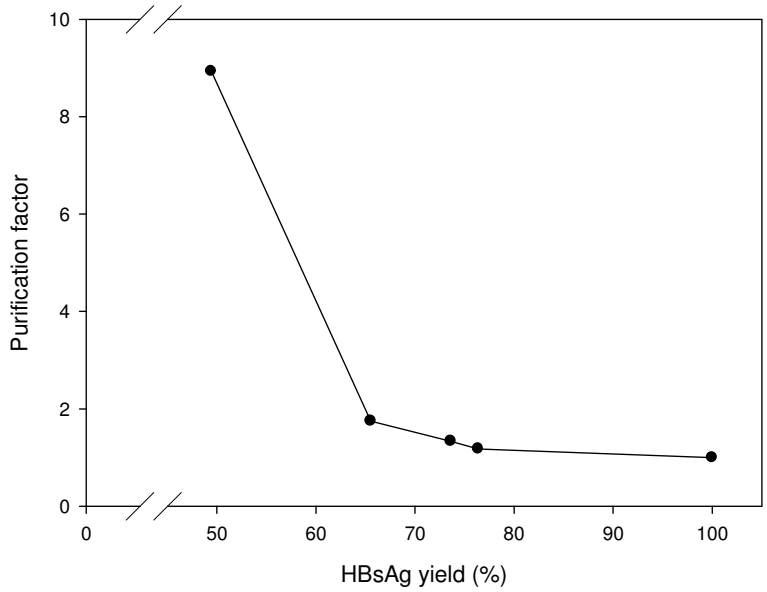


***Figure 5.9**: Trade-off assessment between the levels of purification achieved versus HBsAg yield at different concentrations of ammonium sulphate.*

Precipitation of the average yeast proteins typically requires ammonium sulphate concentrations of above 40% w/v (Van Wijnendaele *et al.*; 1987, Clarkson *et al.*, 1996). Hence, under the ammonium sulphate concentrations investigated here (< 25% w/v) its impact on host protein solubility is expected to be negligible.

In summary, ammonium sulphate precipitation is potentially an effective method for the elimination of host lipid contaminants. To improve product yields, it is vital to reduce the extent of HBsAg co-precipitation and for this purpose, a multifactorial study may be employed to optimise the buffer conditions such as pH, ionic strength and the possibility of using surfactants for this operation.

5.4 Conclusions

The performance of HBsAg purification using PEG and ammonium sulphate as precipitating agents is summarised in **Table 5.1**. Precipitation experiments demonstrated that PEG and ammonium sulphate solutions were both effective as fractionation agents for the HBsAg product. 10% PEG 8000 was found provide the optimal condition for the removal of host proteins with an HBsAg enrichment factor of over 8-fold and a concomitant 2-fold reduction in the level of lipid contaminants. Ammonium sulphate precipitation achieved a 8-fold purification factor relative to lipid contaminants. Separation of protein contaminants is unlikely under the concentrations examined for ammonium sulphate as host proteins would remain in the soluble phase with the HBsAg product. From this study, it appears that PEG would be the preferable precipitating agent for eliminating protein contaminant and ammonium sulphate for host lipid reduction.

This study has highlighted that the main challenge with both precipitation options is in product recovery. For PEG precipitation, separation of the precipitate from the mother liquor using centrifugation is hindered at PEG concentrations $\geq$ 10% w/v due to the increasing viscosity of the mother liquor. This issue could be circumvented to some degree by the use of filtration for precipitate recovery. Some product loss owing to irreversible adsorption of HBsAg to the membrane surface was observed, however this could be minimised by optimising the filtration conditions. The issue with ammonium sulphate precipitation is that substantial amounts of the HBsAg product co-precipitates with the lipid contaminants. Further studies to reduce the extent of co-precipitation would be warranted.

***Table 5.1**: Comparing best achievable precipitation performance from the use of PEG 8000 and ammonium sulphate as precipitating agents for HBsAg purification. Step yield in brackets for PEG 8000 assumes 20% product loss due to irreversible adsorption during filtration. *Host protein reduction is expected to be negligible at 15% w/v ammonium sulphate based on previous findings.*

	PEG 8000	**Ammonium sulphate**
Concentration of precipitant required (w/v)	10%	15%
Reduction in host protein contaminants (%)	90± 5	Negligible*
Purification factor	8-fold	Negligible*
Reduction in host lipid contaminants (%)	65 ± 10	90 ± 5
Purification factor	2-fold	8-fold
Step yield (%)	70 ± 5 (50 ±5)	50 ± 10

Overall, the purification potentials of PEG and ammonium sulphate precipitation demonstrated are promising. The findings in this study could be employed as the foundation for future optimisation studies particularly to fine-tune the precipitation conditions and to improve the efficiency of the separation techniques. Although low yields were observed from the precipitation methods examined, these operations may still be practical provided the levels of contaminant removal achieved facilitate high levels of product yield in subsequent chromatographic steps. Further studies on the benefit of the precipitation steps on high resolution downstream operations would be warranted.

In view of the challenges in HBsAg purification by precipitation an alternative method, which is based on the exploitation of intracellular compartmentalisation characteristics of HBsAg particles on the yeast endoplasmic reticulum (ER), was

explored to impart selectivity to the HBsAg recovery process. This is discussed in the following chapter.

Chapter 6

Selective product recovery for primary purification

6.1 Introduction

As discussed in **Chapter 1**, it has been recognised that in spite of the obvious merits of VLP vaccines, their potentials have yet to be fully realised (Pattenden *et al.*, 2005). One of the main reasons for this is the higher costs of production (Boisgerault *et al.*, 2002) compared to traditional vaccines. The cost in VLP manufacturing, like most protein recovery and purification operations is dominated by the expensive and low throughput chromatographic operations (Przybycien *et al.*, 2004). Although it is unlikely that the use of chromatography can be excluded entirely from the bioprocess route, the ability to reduce the purification burden and to improve their throughput is highly attractive. One of the means of achieving this is by maximizing the potentials of the primary purification stages to reduce contaminants such as host lipids and proteins entering the chromatographic operations.

The typical approach to improving the efficiency of primary purification of VLPs is by the introduction of fractionation techniques such as precipitation using polyethylene glycol (PEG) and ammonium sulphate (Vnek *et al.*, 1977; Tsoka *et al.*, 2000, Peterson *et al.*, 1981). However, as demonstrated in the previous chapter, HBsAg purification was achieved at the expense of product yield. The issue of contamination

removal for lipid-envelope VLPs from *S. cerevisiae* may be addressed from an alternative perspective by drawing upon understandings of the VLP expression characteristics. Lipid-envelope proteins are formed through a budding process from cellular compartments such as the ER, plasma membrane or lipid rafts (Grgacic and Anderson, 2006). Often as in the case of the HBsAg, the VLPs remain permanently localised on the membranous organelles due to the lack of protein transport machinery (Herbert *et al.*, 1956). For product recovery, a detergent extraction step is typically required following cell disruption to release the VLP from the membranous components. The inherent intracellular compartmentalisation of such lipid-envelope VLPs may be exploited for selective product recovery and purification. This concept has been previously adopted in the recovery of proteins from targeted regions such as the periplasmic space of *E.coli* (French *et al.*, 1996; Witholt *et al.*, 1976) and from the cytosol or subcellular organelles in *S. cerevisiae* (Chi *et al.*, 1994; Huang *et al.*, 1991).

This chapter demonstrates how a twin centrifugation strategy with process interaction considerations (between cell disruption and the detergent step) may be applied to exploit the inherent localisation of lipid-envelope VLPs on membranous organelles to impart selectivity to the VLP recovery and purification process. The HBsAg particle is employed as the model VLP in this study. The conventional process route for HBsAg production (Dekleva, 1999; Wampler *et al.*, 1985; Sitrin and Kubek, 1992) which provides the benchmark for the comparative study is as shown in **Figure 6.1 (a)**. The selective product recovery method investigated in this study involves an additional centrifugation step following cell disruption but prior to the detergent-mediated HBsAg liberation **(Figure 6.1 (b))**.

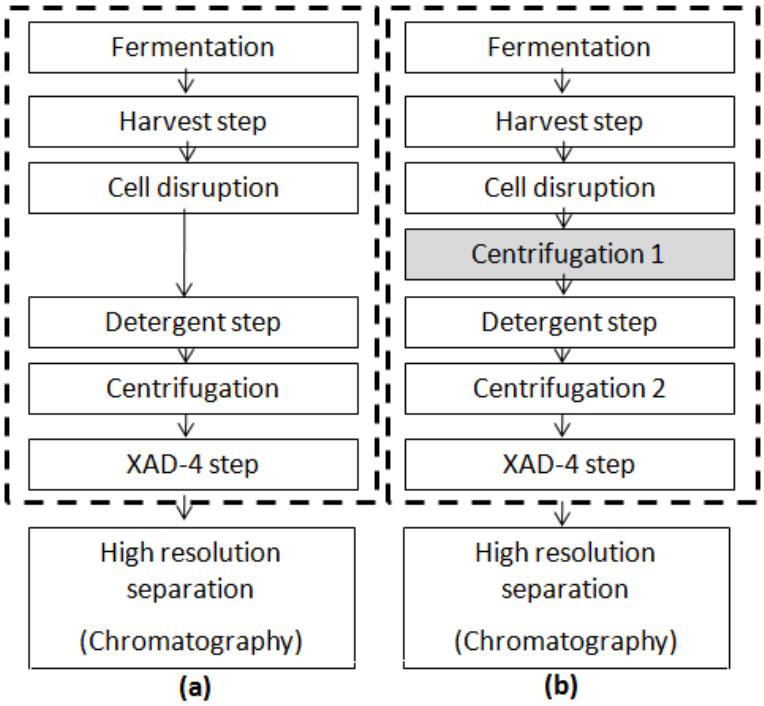


***Figure 6.1**: **(a)** represents the conventional process route for the production of HBsAg as described by Dekleva (1999) and Wampler et al. (1985). **(b)** represents the selective recovery approach investigated which incorporates an additional centrifugation step prior to detergent-mediated HBsAg liberation. (- - -) shows unit operations in primary purification.*

The rationale behind this is that the ER containing the HBsAg product would be sedimented following centrifugation 1 allowing bulk cytosolic material containing significant proportions of yeast proteins, lipid and nucleic acids to be eliminated in the supernatant fraction. Recovery and subsequent treatment of the solids fraction with detergent would then release the HBsAg product into an enriched product stream. A second centrifugation step (centrifugation 2) is then employed for the removal of membranous debris now free of the HBsAg product. The relative partitioning of the HBsAg product and the yeast-derived contaminants between the supernatant and solids fraction following centrifugation 1 was initially evaluated to assess the maximal

achievable yield and degree of purification from this method. To enhance the selectivity of this approach, the impact of homogenisation pressure conditions on product yield and the contamination profile following the detergent step were characterised to identify a suitable process trade-off. Finally, to ascertain the benefits of the proposed purification process, its impact on the performance of a downstream hydrophobic interaction chromatography step was evaluated. The likely merits and process considerations for adopting such a selective product recovery process would aid the development of a process framework for the delivery of future generations of VLP vaccines.

6.2 Materials and Methods

HBsAg material for this study was prepared by fermentation as described in **Section 2.2** and homogenisation was conducted at a range of pressures (100-1200bar) for 4 passes as discussed in **Section 2.9**. The selective product recovery methodology based on a two-step centrifugation approach proposed in this study is detailed in **Section 2.8**. The conventional HBsAg liberation process described in **Section 2.4** was employed as the benchmark for process comparison. The hydrophobic interaction chromatography (HIC) step for analysing the benefits of improved primary purification processing methods on downstream chromatography is detailed in **Section 2.10**.

Sample analysis was performed using an ELISA assay for HBsAg measurement (**Section 2.13.4**), a Bicinchoninic (BCA) assay for total protein determination (**Section 2.13.5**), lipid analysis by HPLC (**Section 2.13.6**) and nucleic acid determination (**Section 2.13.8**). The yeast homogenate following cell disruption were characterised by microscopy as in **Section 2.13.12**.

6.3 Results and Discussions

The objective of this study was to develop a selective product recovery strategy for the purification of lipid-envelope VLPs so as to improve the performance of subsequent downstream chromatography operations. As highlighted in the introduction, the selective recovery approach under investigation is based on the exploitation of VLP localisation characteristics on membraneous organelles in *S. cerevisiae,* requiring the use of a detergent for their release. The method described by Dekleva (1999) and Wampler *et al.* (1985) was used as the benchmark process and VLP yield, purification efficiency and impact on chromatography were used as the criteria for evaluation.

6.3.1 Investigating the potentials of the selective recovery methodology

In the recovery of HBsAg, the use of a detergent is necessary to facilitate the liberation of the VLP from the ER (Dekleva, 1999; Wampler, 1985; Sitrin and Kubek, 1992). As shown in **Figure 6.1(a)**, the detergent is typically added immediately after cell disruption. The HBsAg product is consequently released into a complex process stream containing a mixture of cell debris, host cell organelles, yeast lipid, protein, nucleic acids and other yeast-derived contaminants. In an attempt to reduce the complexity of the process stream, the use of a selective recovery approach as in **Figure 6.1(b)** was investigated.

In this element of the study, homogenisation was carried out at 1200 bar for 4 passes. High homogenisation pressure conditions as such are favoured for rapid and efficient processing (Sitrin and Kubek, 1992). Comparison of the product stream at the end of primary purification generated from the solids fraction using the selective

recovery methodology to that using the conventional method is shown in **Table 6.1**. For mass balance analysis, the level of product loss and the amount of contaminants eliminated in the supernatant waste stream following centrifugation 1 using the selective recovery methodology are also documented.

Table 6.1: *Comparison of the levels of the HBsAg product and major classes of contaminants recovered at the end of primary purification using the conventional method previously described*[22-23] *with the levels observed also at the end of primary purification generated from the solids fraction following the selective recovery methodology. Product loss and the level of contaminants successfully eliminated in the waste stream following the selective recovery methodology are also shown. Measurements reported for total protein and lipid are based on BCA assay and HPLC peak area analysis, respectively. *refers to solids and supernatant streams generated from centrifugation 1.*

	Conventional method	**Selective recovery method**	
		Solids* (Product stream)	**Supernatant* (Waste stream)**
HBsAg (mg/g cell)	8	8	2
Total protein (mg/g cell)	31	9	20
Nucleic acids (µg/g cell)	33	14	42
Total lipid (mAu*mL)	1711	527	1091

ELISA activity measurements revealed that ~ 80% of the HBsAg product was recovered from the solids fraction during centrifugation 1 while the remainder was lost in the supernatant. In spite of some product loss, HBsAg yield from primary purification using the selective recovery methodology at 8 mg/g cell still matched the level attained using the conventional method. This reflects an improvement in the HBsAg liberation efficiency during the detergent step. Given that the detergent typically reacts also with

lipid contaminants, it is not surprising that when such lipids are reduced, enhanced levels of HBsAg liberation by detergent is observed.

Total protein analysis using the bicinchoninic acid (BCA) method estimated that approximately 32% of the total protein remained in the product stream following primary purification. HBsAg purity based on total protein concentration following the selective recovery methodology was calculated at 89% which was a significant improvement over the 26% recorded using the conventional process. The efficiency of host protein reduction was also evident from the SEC profiles in **Figure 6.2**. The HBsAg product has a molecular weight of 3.5 x 10^6 (Gavilanes *et al.*, 1990) which is close to the exclusion limit of the Superose 6 column of 4 x 10^6 (GE product information sheet). The earliest peak (A) in **Figure 6.2** was found to contain the HBsAg product as well as lipids based on the collection of the fraction for ELISA assay and lipid HPLC analysis. Subsequent peaks (B) were due to host cell proteins and the profile for the solids fraction showed a dramatic reduction in these contaminants. [Analysis of HBsAg purity in terms of total protein was also performed. The results however were inconclusive due to the poor resolution of the bands attained.]

Lipid analysis was carried out by HPLC and the profiles are as shown in **Figure 6.3**. The retention time of peak Y matched that of the ergosterol standard while peaks X and Z, based on the chromatogram database from the column manufacturer, were likely due to a sterol and a phospholipid respectively. (Subsequent studies may be required to characterise peaks X and Z.) The chromatograms showed that upon centrifugation, lipids in peak Z were fractionated into the supernatant stream while lipids in peaks X and Y remained in the solids fraction. Since ergosterol or sterols are found mainly in the plasma membrane and secretory vessels (Zinser *et al.*, 1993), it is not surprising that during centrifugation, these lipids were sedimented together with their host organelles.

Overall, a ~70% reduction in total lipid contaminants was achieved in the solids fraction.

In terms of nucleic acid contaminants from the yeast host cell, A_{260} analysis revealed that the level in samples from the solids had been reduced by over 70%. The nucleic acids levels detected in the product fraction and the supernatant waste stream following the selective recovery methodology in total exceeded the level measured from the conventional process. Like in the case with the HBsAg product, this may be a result of the increased rate of nucleic acid extraction by the detergent when a lower level of lipid contaminants is present.

This initial study demonstrates that the inclusion of an additional centrifugation step results in a highly selective recovery methodology with significant potential for the reduction of host contaminants with minimal compromise on product yield. Having established this, subsequent studies were carried out to investigate if the efficiency of contaminant removal achieved by this operation may be further enhanced.

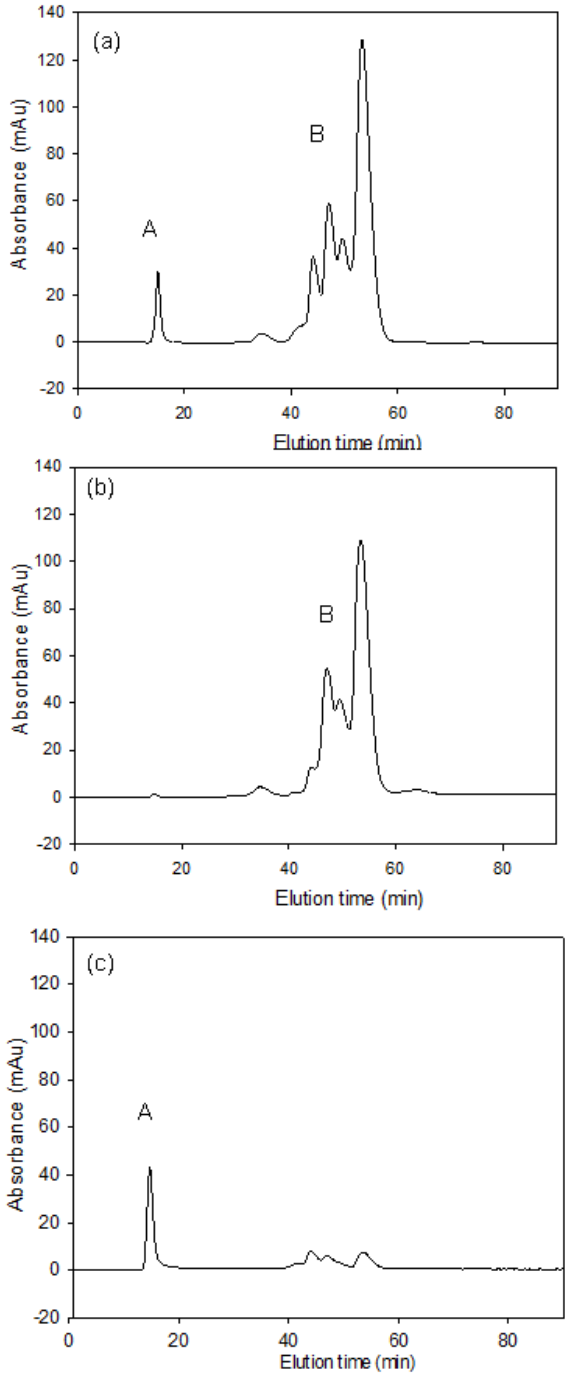


***Figure 6.2**: **(a)** shows the SEC profile of samples at the end of primary recovery following the conventional method as described by Dekleva (1999) and Wampler et al. (1985). **(b)** and **(c)** are profiles of samples at the end of primary recovery obtained from the supernatant and solids fractions, respectively, following the selective recovery approach. Cell disruption for all samples was carried out at 1200 bar for 4 passes.*

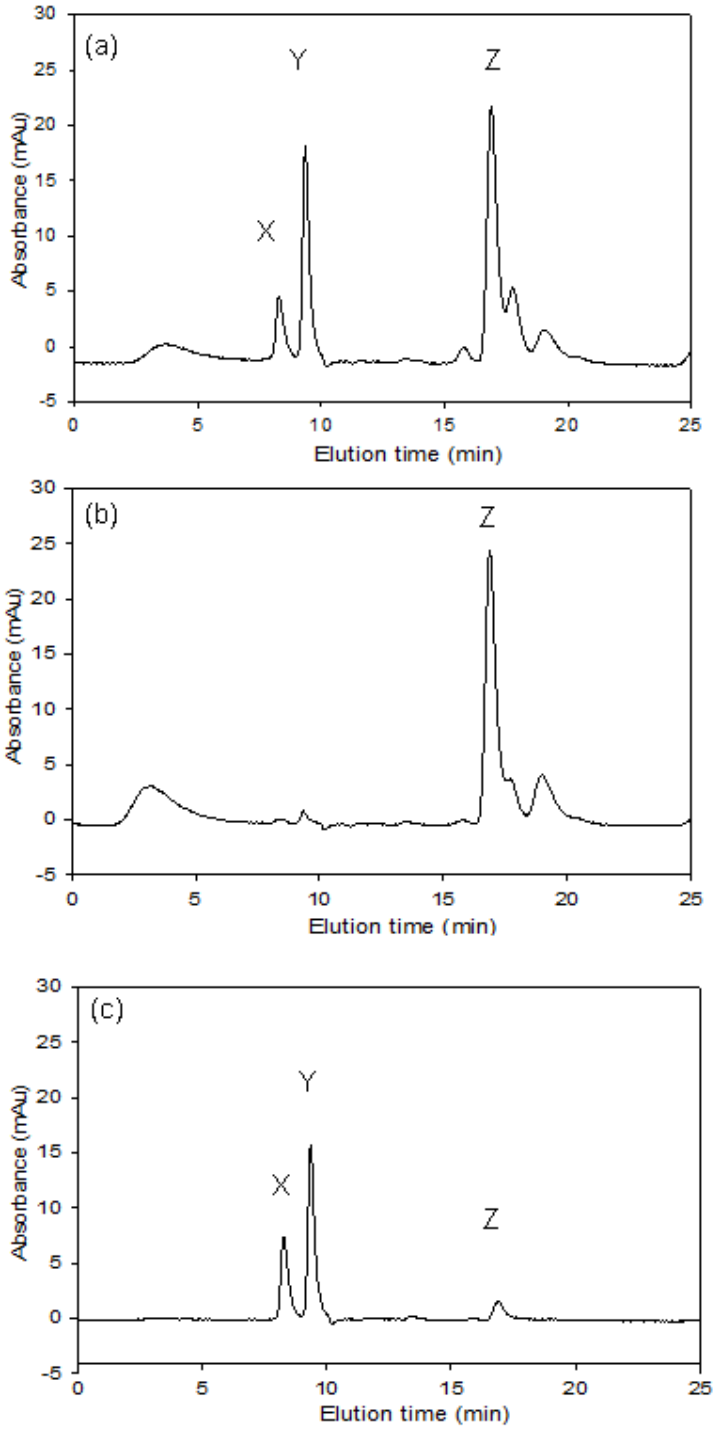


***Figure 6.3**: Chromatogram **(a)** shows lipid profile of samples at the end of primary recovery following the "Deklava" method. Chromatograms **(b)** and **(c)** show lipid profiles obtained at the end of primary recovery from the supernatant and solids fractions following the selective recovery approach. Cell disruption for all samples was carried out at 1200 bar for 4 passes.*

6.3.2 Evaluating the impact of cell disruption conditions on product yield and the level of contaminants

The data for HBsAg recovery and purification up to this point were obtained using homogenisation at 1200 bar, consistent with the process described by Sitrin and Kubek, (1992) for 4 passes, reportedly ideal for HBsAg recovery from yeast (Milburn and Dunnill, 1994; Pointek and Weniger, 2002). Cell disruption conditions, however, do not only impact the level of product recovery but could have an influence on the levels of release of host cell contaminants into the process stream. Hence, studies were carried out to investigate if the proposed selective recovery methodology could be improved by the optimisation of cell disruption conditions.

The selective recovery approach was applied to cell lysate samples generated at different homogenisation pressures conditions (100 – 1200 bar) with a control sample of undisrupted cells. **Figure 6.4** shows the microscopy images of homogenate generated under different disruption pressure conditions. The recombinant *S. cerevisiae* cells for the control sample shows clumps of cells measuring 20 µm in average which is due to the tendency of this strain to aggregate due to the *mnn* mutation (Mendez-Vilas *et al.*, 2004). At 100 bar disruption pressure, some cell breakage and a reduction in the size of cell clumps was observed. Significant cell breakage was observed at 400 bar and above. At homogenisation pressures of 800 bar and 1200 bar, extensive cell fragmentation and aggregating micronised particles were observed.

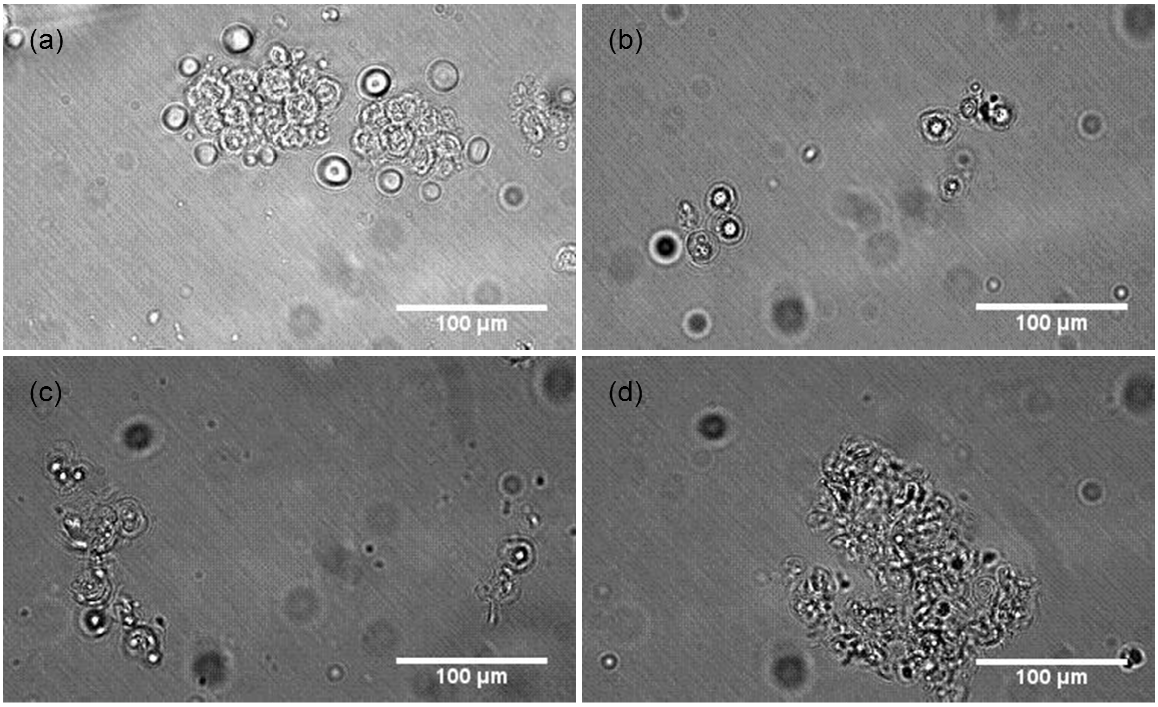


***Figure 6.4**: Microscopy images (1000X magnification) of (a) undisrupted sample, (b) homogenate at 100 bar, (c) homogenate at 400 bar and (d) homogenate at 800 bar.*

Following cell disruption, centrifugation was performed and the solids fractions recovered were treated with detergent. A second centrifugation was carried out for removal of cell debris and the supernatant recovered containing the HBsAg product were analysed in terms of HBsAg yield and the extent of reduction of host cell protein, nucleic acids and lipid contaminants. As shown in **Figure 6.5**, homogenisation pressure conditions had an impact on the level of HBsAg recovery as measured by ELISA. Higher HBsAg recovery was achieved when increasing the homogenisation pressure from 100 bar to 400 bar as greater cell breakage led to more effective release of the VLP during the subsequent detergent step. However, beyond ~ 400 bar, further increases in the homogenisation pressure led to a decrease in the ELISA measurements which is likely to be a reflection of the impact of high shear conditions on HBsAg activity. It is also possible that ER components containing the HBsAg product are more

fragmented at higher homogenisation pressure conditions and as a result the effective sedimentation of these organelles during centrifugation 1 becomes more challenged leading to increased product loss in the supernatant. A substantial amount of the HBsAg was recovered for the control sample that was not homogenised which is surprising. However, this may be due to product leaching as a result of the sample freeze-thaw or the use of detergent which may increase membrane permeability. (Further studies may be required to verify and characterise this phenomenon.)

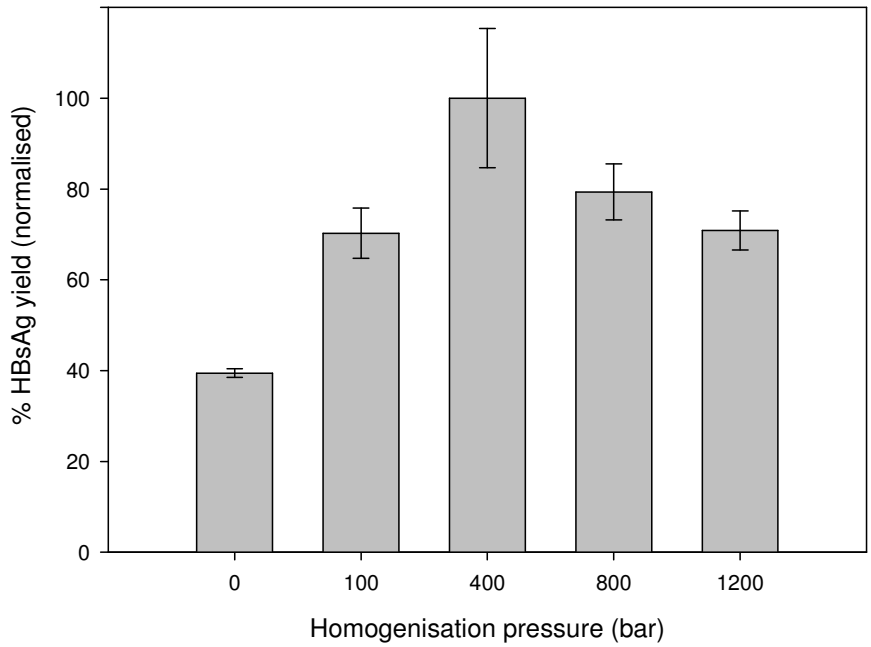


Figure 6.5*: The impact of homogenisation pressure conditions on the level of HBsAg recovery (based on ELISA measurements) from the solids fraction following the selective recovery method. The y-axis was normalised by setting the highest HBsAg recovery level (~10 mg/g cell) to 100%. The error bars represent normalised standard deviation from triplicate samples with calculated coefficient of variance of ≤ 15%.*

Protein and nucleic acid analyses indicated that the effect of homogenisation pressure conditions on the level of the reduction of these contaminants was minimal

(data not shown). Lipid analysis however, yielded interesting results as shown in **Figure 6.6**. The results demonstrated that at high homogenisation pressures, particularly at 800 bar and above, greater release of lipids into the product stream occurred. At higher pressure conditions (≥ 800 bar), the extensive cell fragmentation led to the formation of micronised cell debris which in turn generated larger surface area for the detergent to extract lipids from during the HBsAg recovery step.

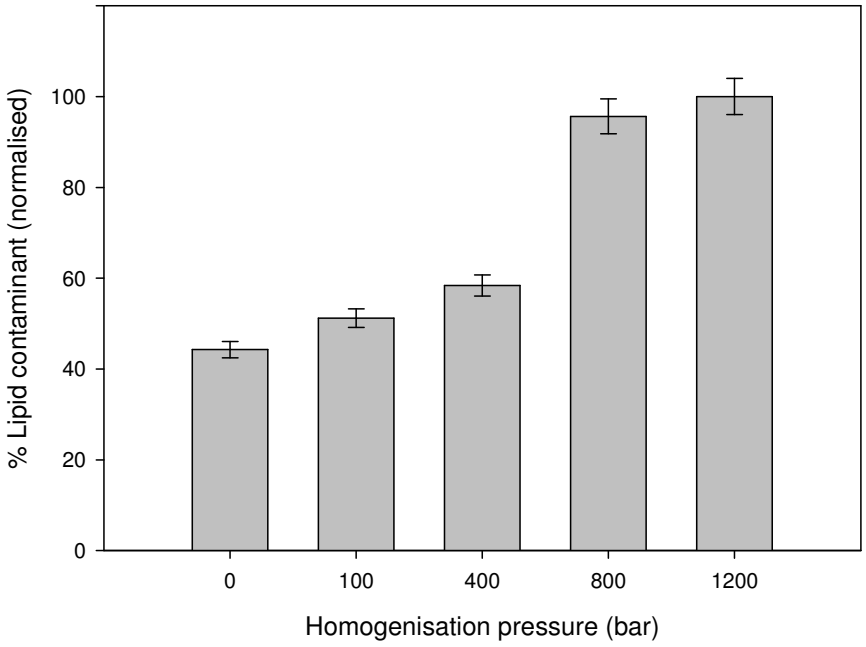


Figure 6.6*: The level of lipid contaminants in the product stream (based on peak area from the lipid HPLC) under different homogenisation pressure conditions following the selective recovery method. The data was normalised by setting the lipid sample with the highest peak area (650 mAu*mL) to 100%. The error bars represent the average coefficient of variance from triplicate samples calculated at 4%.*

The trade-off between product recovery and the efficiency of lipid contaminant removal were explored (shown in **Figure 6.7**). These results suggest that homogenisation pressures in the region of 400 bar are optimal in terms of maximising

the product yield whilst controlling the level of lipid contaminants. The ability to operate at more moderate homogenisation pressure conditions would also be advantageous from the perspectives of lower energy consumption and reduced cooling requirements during the operation.

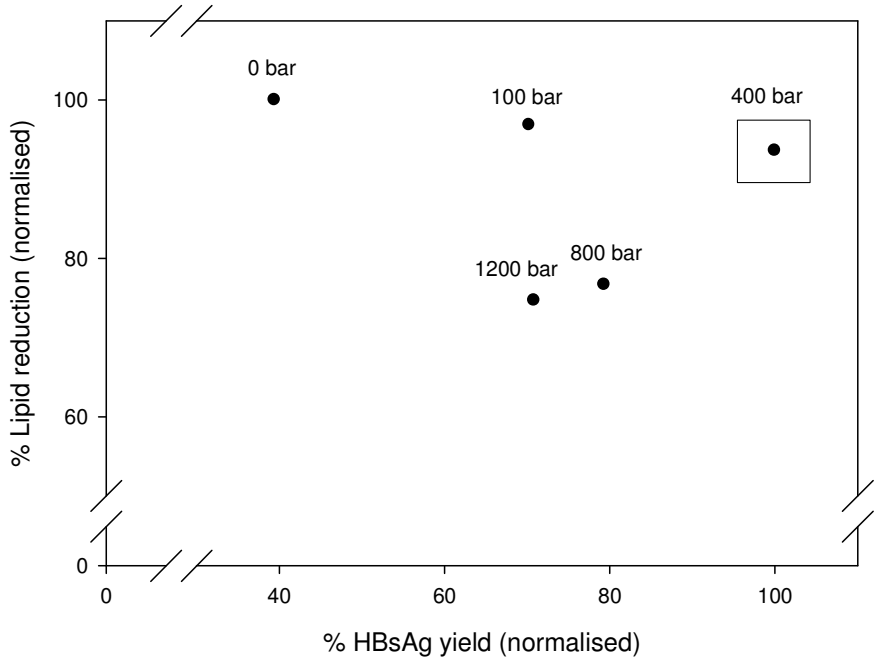


Figure 6.7*: Trade-off considerations of the impact of different homogenisation pressure conditions on the degree of lipid reduction and HBsAg yield at the end of primary purification. (□) shows data point which achieved the best trade-off.*

Investigation on the impact of homogenisation pressure has so far been performed keeping the number of passes constant at 4 as reportedly ideal for VLP recovery from yeast (Milburn and Dunnill, 1994; Pointek and Weniger, 2002) using the same homogenisation set-up. It is indisputable that the number of homogenisation passes and the design characteristics of the homogeniser such as valve seat geometry and impact distance (Miller *et al.*, 2002) could have significant impact on the debris profile thus influencing the level of host protein and lipid contaminants released during the subsequent detergent step. An in-depth study on a range of factors that potentially

impact homogenisation performance may be warranted in order to develop the optimal window of operation for selective product release.

6.3.3 Impact of improved process on HIC performance

To demonstrate the value of the improved process based on the selective recovery methodology and the use of more moderate homogenisation pressure conditions, the resulting impact on the performance of a subsequent hydrophobic interaction chromatography (HIC) operation was investigated. HIC separation is an important chromatography operation in the purification of recombinant HBsAg. Its successful application has been demonstrated by Belew *et al.* (1991). In this study, a HIC challenge was performed comparing three sets of material: (1) material generated using the conventional method (Dekleva, 1999; Wampler *et al.*, 1985) with cell disruption at 1200 bar (sample I); (2) material generated using the selective recovery methodology explored in this work with cell disruption at 1200 bar (sample II); and (3) material generated using the selective recovery methodology examined with cell disruption at 400 bar (sample III).

Fresh columns were employed for each feed material tested. Flow through during the loading stage were collected and analysed for HBsAg using ELISA. Significant proportion of the HBsAg product was measured in the flow through early in the loading stage for sample I. This was also observed for sample II although to a lower extent. Based on the amount of product in the flow through, total HBsAg captured as a proportion of the total amount loaded were calculated and the results are summarised in **Table 6.2**. This is shown graphically in **Figure 6.8**, where the cumulative amounts of HBsAg captured in relation to the cumulative amounts fed to the column at different

time points during the loading stage are plotted. The diagonal line (slope = 1) indicates 100% product capture. The profile for sample III which is closest to the line of 100% product capture suggests that this feed stream achieved the highest level of product binding relative to the amount loaded. This is not surprising given that this feed material had the lowest levels of host protein and lipid contaminants. Lower levels of contaminants in the chromatography feed would reduce competitive binding and non-specific interactions with the resin thereby ensuring greater binding capacity for the product of interest. Consistent with this, Sample II, which was substantially cleaner than sample I owing to the use of the selective recovery procedure but lower in purity than sample III due to the higher homogenisation pressure employed, showed an intermediate level of product binding relative to the amount loaded.

Table 6.2*: Summary of the results from the HIC challenge for the process streams generated using the different methodologies investigated. Amounts of HBsAg were measured by ELISA assay.*

	Conventional method	**Selective recovery method**	
	Homogenisation at 1200 bar (Sample I)	**Homogenisation at 1200 bar (Sample II)**	**Homogenisation at 400 bar (Sample III)**
HBsAg captured as a proportion of HBsAg loaded (%)	38	56	78
HBsAg eluted as a proportion of HBsAg captured (%)	97	95	98
Overall step yield (%)	37	53	77

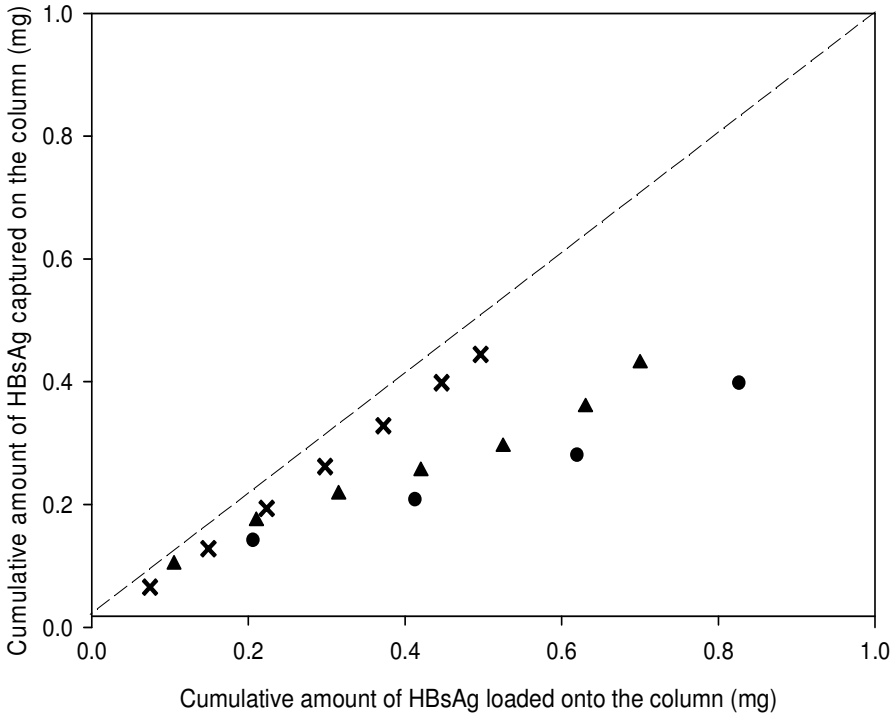


***Figure 6.8**: Cumulative amount of HBsAg product captured on the column relative to the cumulative amount loaded for material generated using the conventional process (Dekleva, 199; Wampler et al., 1985) with homogenisation pressure of 1200 bar (sample I) (●); for material generated using the selective recovery method with homogenisation pressure of 1200 bar (sample II) (▲); and for material generated using the selective recovery method with homogenisation at 400 bar (sample III) (✕). (-----) indicates 100% capture of the HBsAg product loaded.*

Analysis of the elution fractions in **Table 6.2** showed that elution conditions were effective as the elution step achieved almost complete recovery (≥ 95%) of the HBsAg material bound to the column during the product loading stage for all samples. As expected Sample III, which had the highest proportion of product captured by the column also achieved the highest step yield at 78%. SEC and lipid HPLC analyses showed that the elution fractions were comparable in terms of product purity indicating that the HIC resolution is independent of the level of contamination in the feed stream.

It is however important to note that in this study, new columns were employed for each run. The rate at which the column resolution deteriorates as a result of fouling after multiple cycles of usage may differ for the feed streams investigated. Further studies would be required to quantify this.

6.4 Conclusions

The effective recovery and purification of detergent-liberated intracellular recombinant VLPs, such as the hepatitis B surface antigen (HBsAg), is exceedingly challenging due to the high levels of co-existing host-derived contaminants. In the bioprocessing of such high-value low titre products, the prerequisites are high yield, purity and maintenance of product integrity. This work has demonstrated the potential of a selective product recovery methodology which capitalises on the inherent compartmentalisation of lipid-envelope VLPs on membraneous organelles for effective primary purification. Understanding of process interactions between cell disruption and detergent-mediated product recovery is vital in ensuring optimal trade-off for high VLP yield with minimum co-extraction of host-derived contaminants. The benefit of the proposed primary purification strategy on a downstream hydrophobic interaction chromatography step is evident. As a result of the elimination of bulk contaminants in the process stream, a significant improvement in column binding capacity and a concomitant increase in product yield were achieved. Furthermore, the reduction in the level of contaminants, particularly lipids, would serve to minimise column fouling thereby improving column integrity and lifespan. As such, the selective recovery

methodology described here may provide a platform for future VLP process developments.

The unit operations employed in this selective process are essentially the same as for a conventional process but with the inclusion of an additional centrifugation step. The effectiveness of this selective recovery approach depends on the efficiency of the centrifugation steps in the process sequence, particularly the levels of dewatering and clarification achieved. The feasibility of this operation for large scale VLP manufacturing would hence be dictated by centrifugation performance upon scale-up. Scale-up considerations for the key centrifugation operations are discussed in the following chapter.

Chapter 7

Centrifugation scale-up studies for the selective product recovery methodology

7.1 Introduction

In the previous chapter, an enhanced primary purification methodology for the recovery of the hepatitis B surface antigen (HBsAg) was developed based on the introduction of a key centrifugation step post-cell disruption but prior to the addition of detergent for HBsAg liberation from the endoplasmic reticulum (ER). The two-step centrifugation process is shown schematically in **Figure 7.1** and the centrifugation steps are denoted as centrifugation "1" and "2" respectively. The purpose of the first centrifugation step (centrifugation 1) is to recover sedimented cell membrane material containing the HBsAg product of interest and to eliminate bulk cytosolic host protein and lipid contaminants in the supernatant. Hence, this centrifugation step influences the product yield and degree of product enrichment achieved. Following this, the sedimented fraction is treated with Triton X-100 detergent to release the HBsAg product into the soluble phase. A second centrifugation step (centrifugation 2) is then employed for the removal of cell debris, now free of HBsAg. Since the HBsAg product is recovered in the supernatant fraction, the degree of dewatering is critical to achieve the desired product yield. This twin centrifugation methodology has been successfully demonstrated at laboratory scale. However, successful translation of this strategy to

large scale manufacturing would depend on the dewatering and clarification performance of the key centrifugation operations upon scale-up. Hence, it is vital that the HBsAg yield and the efficiency of contaminant removal observed at laboratory scale are not lost in the scaled up process.

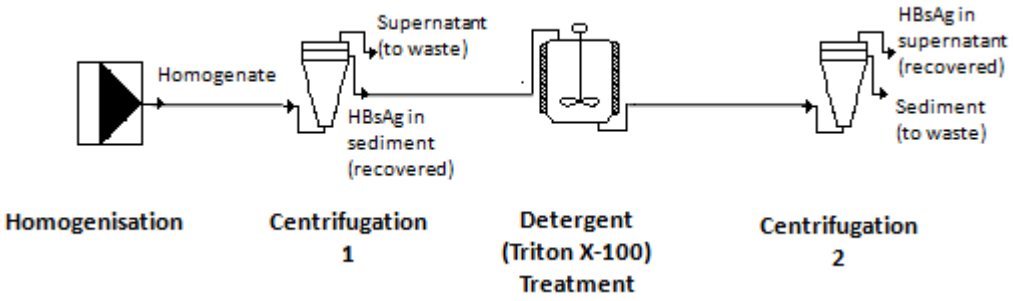


***Figure 7.1**: Schematic of two step centrifugation process adopted in the proposed selective product recovery methodology for the primary purification of HBsAg.*

The ability to predict large scale centrifugation performance using scale down methods is invaluable from both the perspectives of time and costs. The concept of ultra scale-down (USD) centrifugation using millilitre quantities of material, developed in recent years has shown enormous potential in predicting the performance of large scale industrial operations (Tustian *et al.*, 2007; Boychyn *et al.*, 2004; Boychyn *et al.*, 2000; Salte *et al.*, 2006; Titchener-Hooker *et al.*, 2008). USD predictions are based on the Sigma concept (Ambler, 1959) which infers that maintenance of equivalent flow to separation area ratios will result in the same level of clarification being achieved for different centrifuges. Correction factors are introduced to account for non-ideal flow patterns and these differ depending on the type of centrifuge. An additional stage is usually involved when modelling the recovery of delicate materials. By the inclusion of a shear device, it is possible to mimic the impact of high-shear regions present in the inlet of centrifuges which may cause cell disruption and hence lead to a carryover of

solid contaminants (Boychyn *et al.*, 2004; Tustian *et al.*, 2007). This is not required when dealing with post-homogenisation material, such as in this study, where the effects at high shear regions of the centrifuge would be insignificant compared to cell disruption conditions to which the material will have had prior exposure.

A wide range of centrifuge designs are available for large scale processing. Amongst these are the disc-stack centrifuge, the multichamber bowl and a tubular bowl variant, the CARR Powerfuge™. Their characteristics have been previously reported (Salte *et al.*, 2006; Boychyn *et al.*, 2004). The CARR Powerfuge™ is of particular relevance since this high-speed industrial centrifuge, being similar to the tubular bowl in design, provides good clarification and solids compaction but crucially is also capable of automated solids discharge. It thus possesses the combined advantages of the tubular bowl and disc-stack machines (Boychyn *et al.*, 2004). The CARR Powerfuge™ P6 model for process demonstration has an operating feed flow range from 0.1 to 1 L/min and rotational speed of 5,000 to 15,000 rpm.

The aim of this study was two-fold. The fundamental goal was to demonstrate the scalability of the selective product recovery approach, specifically that its benefits are not compromised upon scale-up. The secondary goal was to investigate if centrifugation conditions could be tuned to enhance further the selectivity of contaminant removal using this method. The first step to both goals involves determining the optimal range of operating conditions for the two centrifugation steps. Ultra scale-down (USD) centrifugation experiments were adopted to mimic conditions of feed flowrate and rotational speed within the operating range of the CARR Powerfuge™ P6 centrifuge. Centrifugation performance were analysed in terms of the degree of solids recovery, the clarification level, percentage dewatering, HBsAg yield and the effective removal of host protein and lipid contaminants. A pilot-scale study

was performed to evaluate the "optimal" and "worst case" scenarios identified from the USD mimics. The pilot-scale study would allow the assessment of the accuracy of the USD centrifugation predictions and more importantly for the verification of the scalability of the selective product recovery methodology.

7.2 Theoretical considerations

USD centrifugation theory is based on the Sigma concept (Ambler, 1961) which allows the separation area of a centrifuge to be described independently of process parameters. The sigma value, ∑, can be used as an index for the comparison of centrifuges of different types or for the prediction of the performance of a given centrifuge during scale up as shown in **Equation 7.1** where Q is the feed flowrate into the centrifuge, ∑ the equivalent settling area and C the correction factors to account for nonidealities in fluid flow.

$$\frac{Q_1}{C_1\Sigma_1} = \frac{Q_2}{C_2\Sigma_2} = \frac{Q_3}{C_3\Sigma_3} \qquad \text{(Equation 7.1)}$$

Different centrifuge designs result in different expressions for the settling areas. In this study, the performance of a CARR Powerfuge™ tubular bowl type centrifuge was modelled using a laboratory fixed angle centrifuge. The equivalent settling area, ∑, for the CARR Powerfuge can be calculated using the tubular bowl **Equation 7.2** whereby ω is the angular velocity, g, gravitational acceleration, L, bowl length, r_o, outer bowl radius and r_1, radius of weir for supernatant discharge (Boychyn *et al.*, 2000).

$$\Sigma = \left(\frac{\omega^2}{g}\right) \pi L \left[\frac{r_o^2 - r_1^2}{ln\left(\frac{2r_o^2}{r_o^2 + r_1^2}\right)}\right] \qquad \text{(Equation 7.2)}$$

[The CARR Powerfuge^{TM} P6 centrifuge (Pneumatic Scale Corporation, Florida, USA) employed in this study has the following dimensions: L = 0.1273m; r_0 = 0.0762m and r_1 = 0.0508 m.]

The sigma value for the laboratory centrifuge can be calculated using **Equation 7.3** whereby V_{lab} is the volume of the material in the tube and R_1 and R_2 are the respective distances between the centre of rotation and the top of the liquid and the bottom of the tube (Boychyn *et al.*, 2000).

$$\Sigma_{\text{lab}} = \frac{V_{lab}\omega^2}{2gln\left(\frac{2R_2}{R_2+R_1}\right)} \qquad \text{(Equation 7.3)}$$

[Using the Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany) (with Eppendodf tubes filled to 1.5 mL), the following parameters were employed for calculating the sigma value: R_1 = 0.06875 m; R_2 = 0.11307 m.]

The laboratory centrifuge was used as the baseline for comparison hence the correction factor was set at 1.0. The CARR centrifuge is reported to have a corresponding correction factor of 0.9 (Boychyn *et al.*, 2004).

7.3 Materials and Methods

Recombinant *S. cerevisiae* cells expressing the HBsAg product were produced by batch fermentation as reported in **Section 2.2**. For the ultra scale-down study, cell homogenate was prepared by cell disruption using the Lab 40 homogeniser (APV Gaulin GmbH, Lubeck, Germany) at 400 bar for 4 passes as detailed in **Section 2.3**. Ultra scale-down experiments to study the impact of varying centrifuge rotational speed

and feed flow rate are described in **Section 2.11**. Following the ultra scale-down experiments, a validation study was performed at pilot-scale. Due to the lack of availability of recombinant *S. cerevisiae* for this study, baker's yeast (Craftbake High Activity, DCL Yeast Ltd, Surrey, UK) was employed. Homogenisation for this scale of operation was performed using the Lab 60 homogeniser (APV Gaulin GmbH, Lubeck, Germany) under the same conditions as for the USD study of 400 bar and 4 passes. The operation of the pilot scale centrifugation experiment is discussed in **Section 2.12**.

Analytical techniques performed for characterisation of centrifugation conditions include analysis of solids recovery or clarification level (Section 2.13.13), analysis of the dewatering level (Section 2.13.4), HBsAg measurement via an ELISA assay (**Section 2.13.4**), total protein analysis using a Bicinchoninic (BCA) assay (**Section 2.13.5**), and lipid analysis by HPLC (**Section 2.13.6**).

7.4 Results and discussions

The CARR Powerfuge^{TM} P6, which is a pilot-scale centrifuge with a 1 L bowl volume was employed as the basis for the assessment of the scalability of the selective recovery methodology. As previously mentioned, this centrifuge has an operating range of 5,000 to 15,000 rpm in terms of rotational speed and feed flowrate of 0.1 to 1 L/min. Based on these values, the Q/C$\sum$ range for this centrifuge was calculated to be between 1.86 x 10^{-9} to 1.67 x 10^{-7} m/s. Ultra scale-down experiments were conducted to mimic centrifugation performance within the operating range of the CARR Powerfuge^{TM} P6 using recombinant *S. cerevisiae* homogenate. As previously discussed, the two centrifugation operations have significantly different roles and were evaluated

independently. Having identified the optimal centrifugation conditions using USD studies, pilot-scale experiments were performed to verify the accuracy of the USD predictions and to assess the overall scalability of the selective product recovery methodology. This was carried out using a parallel system with baker's yeast due to the lack of availability of *S. cerevisiae* material containing the recombinant HBsAg product for this scale of operation.

7.4.1 Ultra scale-down studies of centrifugation 1

As previously discussed, the role of centrifugation 1 is to achieve effective purification by driving the partitioning of HBsAg-ER material to the solids fraction and the bulk contaminants to the supernatant fraction. In order to determine suitable centrifugation conditions for this step, laboratory centrifugation was carried out under a range of rotational speeds and centrifugation times to achieve a Q/C$\sum$ range of 1.28 x 10^{-9} to 1.8 x 10^{-7} m/s which encompasses the operating range of the pilot-scale centrifuge. The resulting level of solids recovery and percentage dewatering are as shown in **Figure 7.2**. Higher levels of solids recovery and dewatering were observed at lower Q/C$\sum$ conditions which are as expected.Lower Q/C$\sum$ values correspond to the more extreme centrifugation conditions which would in turn lead to more effective settling and compaction of cell debris particles. It was observed that even at the upper Q/C$\sum$ operating limit of the CARR centrifuge, the centrifugation conditions were sufficient to sediment ~ 80% of the solids although the level of dewatering was significantly poorer at ~ 45%.

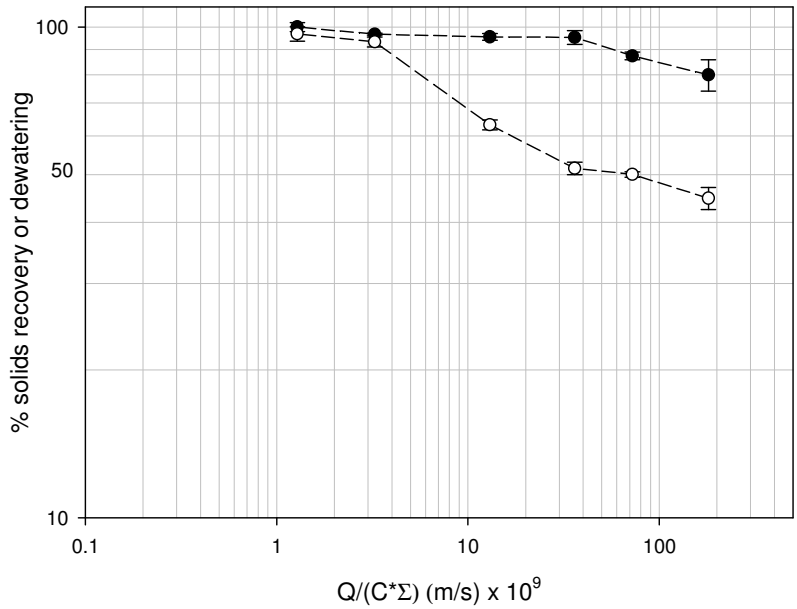


***Figure 7.2**: Level of solids recovery (—●—) and dewatering (—○—) observed for the centrifugation 1 under different Q/C∑ conditions. The results were normalised based on values for the well spun sample set to 100%. Error bars represent standard deviation from triplicate samples with a coefficient of variance ≤ 5%.*

The wet pellets obtained from centrifugation 1 were subsequently treated with detergent for the recovery of the HBsAg product. A second centrifugation step was necessary for the recovery of soluble product for analysis and this was performed at a constant Q/C∑ (1.3×10^{-9} m/s) conditions for all samples. An ELISA assay employed to detect HBsAg antigenicity in the product stream following both centrifugation operations showed little variation in its measurements across the Q/C∑ range investigated for the first centrifugation step (**Figure 7.3 (a)**). The almost identical HBsAg yields are not surprising given that there is less than 20% difference in the solids recovery level over the Q/C∑ range examined. Variations in HBsAg yields, if any, may have been undetectable using the ELISA assay which has a coefficient of variance of up to 17%.

The product streams were also analysed in terms of the level of proteins and lipids present. **Figure 7.3(b)** shows the total protein measurements for the product stream following both centrifugation operations and that for the waste supernatant stream from centrifugation 1 over the range of Q/C∑ examined for centrifugation 1. Since the protein contribution from the HBsAg product appears to be constant over the Q/C∑ range from the ELISA results, variations in total protein measurements must be due to variations in the level of host proteins. The results indicate that higher Q/C∑ conditions (less extreme centrifugation) were beneficial as this lead to greater levels of protein contaminants being eliminated in the waste supernatant stream. As a result the HBsAg product was released into a cleaner process stream following the detergent step. In contrast under low Q/C∑ conditions (more extreme centrifugation), significant proportions of protein associated cell debris co-sedimented with the HBsAg product leading to higher levels of co-liberation of protein contaminants during the detergent step. Total lipid analysis of the product stream following both centrifugation operations is shown in **Figure 7.3(c)**. The results mirror those obtained for the protein contaminants. More effective elimination of lipid contaminants in the supernatant fraction was achieved using higher Q/C∑ conditions for centrifugation 1.

In summary, higher Q/C∑ values are favoured for the operation of centrifugation 1 since more effective removal of host protein and lipid contaminants were achieved. Although dewatering levels were lower under these conditions, which resulted in more dilute feed streams and larger processing volumes for subsequent operations, the benefits of a cleaner process stream would probably outweigh this. Hence, it would be ideal to operate at the Q/C∑ upper limit for the CARR Powerfuge^{TM} which at 1.6 x 10^{-7} m/s is achieved when the machine is operated at a feed flowrate of 1 L/min and a rotational speed of 5000 rpm.

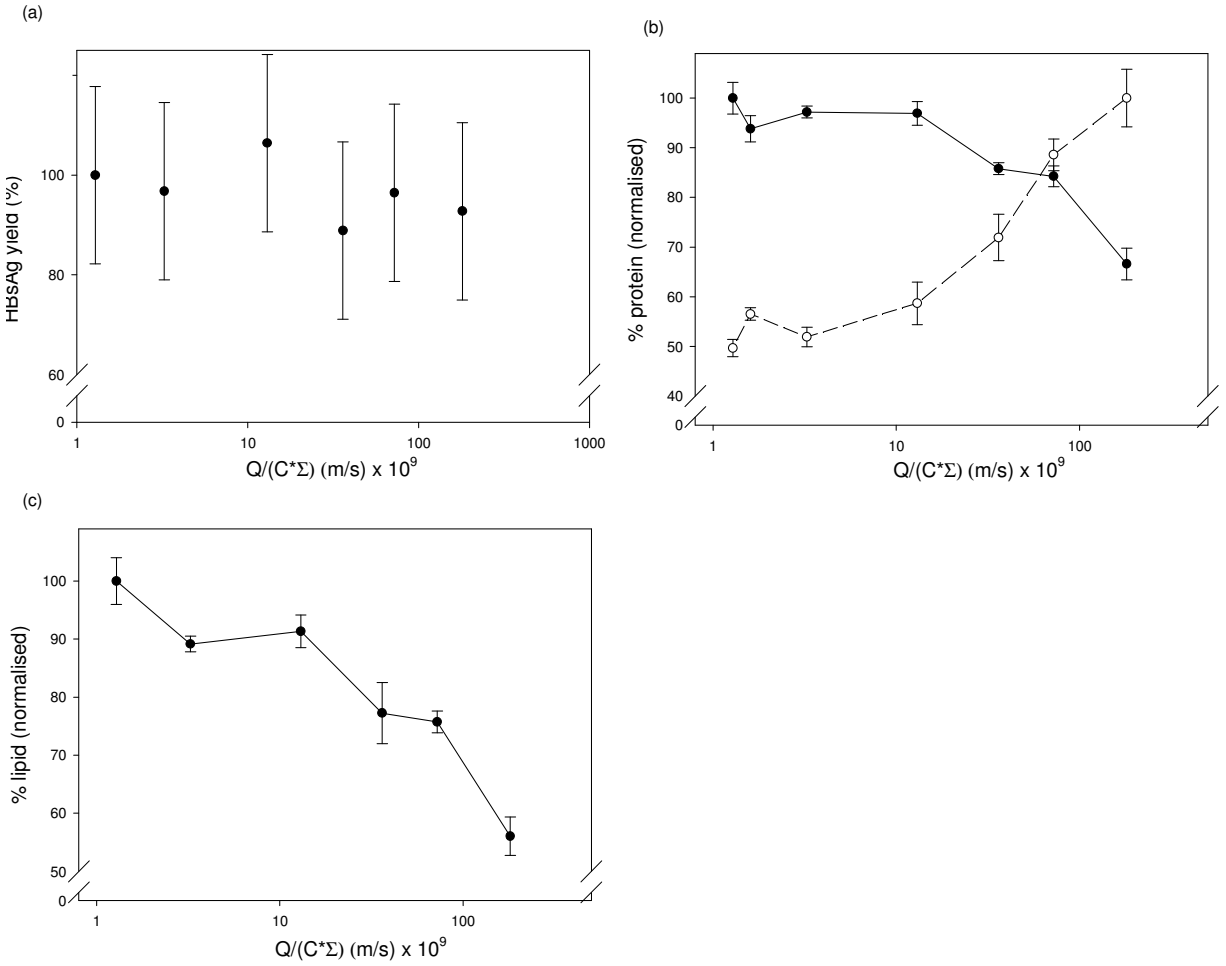


Figure 7.3*: (a) shows HBsAg yield following the detergent step generated under different Q/C∑ conditions employed for centrifugation 1. (b) shows the protein levels eliminated in the waste stream following centrifugation 1 (—○—) and the protein levels in the final product stream (—●—). (c) shows the level of lipid contaminants in the final product stream under conditions for centrifugation 1. Error bars represent standard deviation from triplicate samples with average coefficient of 17% for HBsAg measurement by ELISA and coefficient of variance of ≤ 5% for protein and lipid measurements.*

7.4.2 Ultra scale-down studies of centrifugation 2

The role of centrifugation 2 is to recover the HBsAg product now in the supernatant fraction. Hence, high dewatering performance is critical to maximise product yield. HBsAg-enriched cell debris sediments were prepared centrifugation 1 using the optimal Q/C∑ value of 1.6 x 10^{-7} m/s determined previously. Following detergent-mediated HBsAg liberation, centrifugation 2 was performed under a range of Q/C∑ conditions of 1.3 x 10^{-9} to 6.5 x 10^{-7} m/s.

Figure 7.4 shows the impact of Q/C∑ on the level of clarification and dewatering from centrifugation 2. As in centrifugation 1, it was observed that lower Q/C∑ conditions (more extreme centrifugation) led to higher levels of solids recovery and dewatering in centrifugation 2. Since the product of interest is recovered in the supernatant stream high dewatering conditions are critical and hence operating at the lower Q/C∑ limit of the machine (more extreme centrifugation) would be preferred.

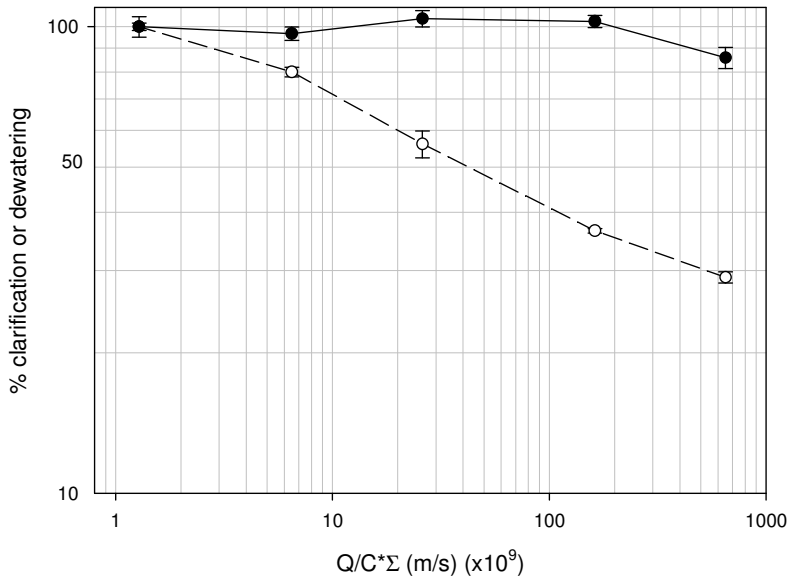


***Figure 7.4**: Level of clarification (—●—) and dewatering (— -○- —) achieved for the centrifugation 2 under different Q/C∑ conditions. The results were normalised by comparison with values for the well spun sample set to 100%. Error bars represent standard deviation from triplicate samples with coefficient of variance of ≤ 5%.*

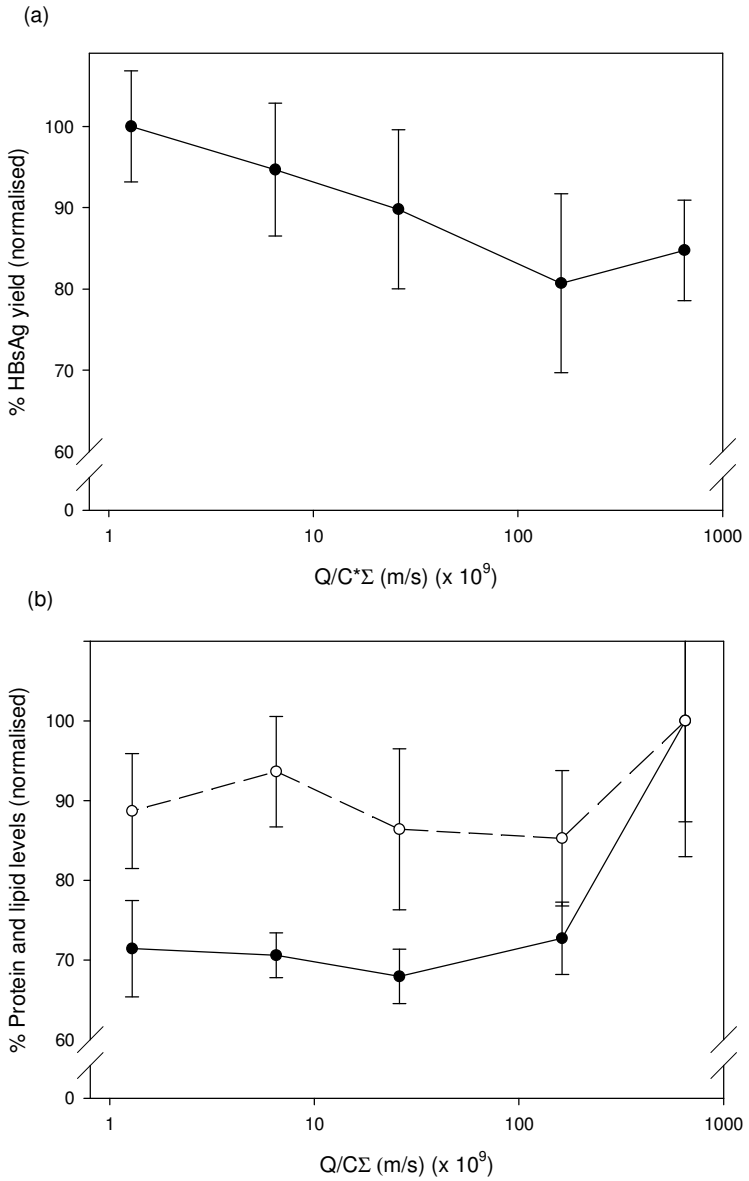


***Figure 7.5**: (a) Impact of centrifugation Q/C∑ conditions on the level of HBsAg product recovery; (b) Impact of centrifugation on the level of protein (—●—) and lipid contaminants (—○—) in the final product stream. Error bars represent standard deviation from triplicate samples with an average coefficient of variance of ≤ 17% for HBsAg measurements and ≤ 5% for protein and lipid measurements.*

Improvement in HBsAg yields was evident from the higher dewatering levels achieved at low Q/C∑ (more extreme centrifugation) conditions as shown in **Figure 7.5 (a)**. This is primarily due to the larger volume of product stream recovered under such conditions. Analysis of the product stream centrifugation 2 in terms of total protein and

lipid are presented in **Figure 7.5(b)**. The use of low Q/C∑ centrifugation conditions also showed up to a 20% improvement in the reduction of protein contaminants. However it did not appear to have a statistically significant effect on the level of lipid contaminants.

7.4.3 Pilot-scale evaluation of the USD predictability

The ultra scale-down (USD) experiments have allowed the rapid identification of the optimal operating conditions for each of the centrifugation steps. It appears that the optimal centrifugation strategy using the pilot-scale CARR Powefuge™ P6 centrifuge would involve operating centrifugation 1 at the high Q/C∑ limit achieved at a feed flowrate of 1 L/min and rotational speed of 5000 rpm, and centrifugation 2 at the low Q/C∑ limit of the machine, at feed flowrate of 0.1 L/min and rotational speed of 15,000 rpm. A pilot-scale study was subsequently performed to assess the accuracy of the USD predictions. In this study, two sets of centrifugation conditions representing the "optimal" and "worst case" centrifugation scenarios were tested and the results compared to those from a USD mimic.

The pilot-scale study would require up to 2 kg of wet cell paste. A fermentation at 450 L scale was carried out to generate material for these studies however the fermentation encountered contamination problems which could not be rectified within the time period allocated for this study. The study was performed instead using baker's yeast as a parallel system. For process comparability, the USD study was also carried out using baker's yeast. The degree of predictability of pilot-scale performance using USD methods determined here for baker's yeast would be reflective of the predictability

of USD methods for the recombinant yeast strain. The use of baker's yeast would allow key parameters such as the level of solids recovery and percentage dewatering to be determined. The contamination profile in the product and waste streams observed here would be indicative of the levels expected in the purification of recombinant HBsAg. Although it is not possible to directly analyse the level of HBsAg yield using this baker's yeast system, an estimation of this is possible from the correlation of product yield with the level of solids recovery and dewatering from centrifugation 1 and 2, respectively. Previous data on HBsAg recoverability from the solids fraction is available from **Chapter 6**.

Comparison of the results obtained at pilot-scale and for the USD mimic for the "optimal" and "worst case" centrifugation strategies are as summarised in **Table 7.1**. In terms of solids recovery, it appears that for higher $Q/C\sum$ (less extreme centrifugation) conditions, the amount of solids recovered were significantly lower than the levels predicted from the USD mimic. For example, in centrifugation 1 of the "optimal" method, the actual amount of solids recovered from centrifugation discharge at 54% was significantly lower than the 96% solids recovery level predicted from the USD method. The supernatant fraction was subsequently analysed to determine if the solids were lost in the supernatant due to poor clarification. The level of solids which were carried over to the supernatant stream was found to only account for 11% of the total solids in the feed. It was observed during centrifugation discharge that some proportion of the solids escaped the centrifuge scraper mechanism and remained on the surface of the bowl. By mass balance, this was estimated to account for ~ 35% solids loss. If the scraper mechanism for solids discharge had been effective and there had been no product lost on the centrifuge bowl, the level of solids recovery would be ~ 90% and significantly closer to the USD predicted value of 96%. Similarly, if product loss due to

ineffective solids discharge was negligible for centrifugation 2 for the "worst case" scenario, the solids recovery level would be at 98%, close to the predicted value of 100% from the USD model.

This effect was less pronounced for centrifugation processes at the low $Q/C\sum$ (more extreme centrifugation) conditions in which the USD predictions more closely matched the actual amounts of solids recovered. The main reason for this is that under more extreme centrifugation conditions at low $Q/C\sum$ values, the solids formed are drier, less sticky and therefore could be removed more effectively from the bowl by the centrifuge scraper mechanism during discharge.

The amount of product loss due to the inefficiency of the scraper mechanism as a proportion of the maximum recoverable solids would be expected to vary depending on the scale of the process. Hence, this loss would be less significant for industrial scale centrifugation processes where larger throughputs are involved. The results imply that the USD model is able to predict accurately the degree of clarification achieved in the supernatant fraction but is more limited in its capacity to predict the actual amount of solids recovered as this depended on the efficiency of the scraper mechanism of the centrifuge in discharging the accumulated solids.

For predictions of dewatering levels, a larger discrepancy was observed between pilot-scale and USD data. During the pilot-scale centrifugation runs, it was observed that the solids discharged were not homogenous. A wet slurry was typically released first followed by the discharge of drier solids. Due to the lack of homogeneity of the discharged material, it was difficult to recover a representative sample for the dry cell weight analysis required in determining the level of dewatering achieved. The driest portions of the solids discharged were relatively easy to sample for dewatering analysis

and this is reflected in the results obtained (**Table 1**) as higher dewatering measurements were recorded for pilot-scale centrifugation runs compared to the USD mimic.

Samples from the supernatant fractions were also measured in terms of the level of protein and lipid contaminants eliminated in the supernatant fractions from centrifugation 1 and the levels remaining in the supernatant product stream from centrifugation 2. The results summarised in **Table 7.2**, clearly show that the levels of protein and lipid contaminants predicted for each of the process streams closely reflect the actual values recorded from the pilot-scale operations.

Table 7.1*: Comparison of the levels of solids recovery and dewatering obtained under "optimal" and "worst case" centrifugation conditions for the pilot-scale experiment and the ultra-scale down (USD) mimic. Values of solids recovery in brackets were determined assuming 100% efficiency of solids discharge.*

	"Optimal"		"Worst case"	
	USD mimic	Pilot-scale	USD mimic	Pilot-scale
Centrifugation 1				
Condition	$Q/C\sum = 1.7 \times 10^{-7}$ m/s		$Q/C\sum = 1.9 \times 10^{-9}$ m/s	
Solids recovery	96%	54% (89%)	100%	96%
Dewatering	76%	96%	100%	87%
Centrifugation 2				
Condition	$Q/C\sum = 1.9 \times 10^{-9}$ m/s		$Q/C\sum = 1.7 \times 10^{-7}$ m/s	
Solids recovery	97%	96%	100%	76% (98%)
Dewatering	97%	100%	73%	97%

***Table 7.2**: Comparison of contamination profile of supernatant streams following centrifugation 1 and 2 for the pilot-scale operation and the USD mimic of the "optimal" and "worst case" centrifugation scenarios.*

	"Optimal"		"Worst case"	
	USD prediction	Pilot-scale data	USD prediction	Pilot-scale data
Centrifugation 1: Contaminants eliminated in supernatant				
Condition	$Q/C\sum = 1.7 \times 10^{-7}$ m/s		$Q/C\sum = 1.9 \times 10^{-9}$ m/s	
Total protein (mg/mL)	5.7	5.8	5.4	5.1
Total lipid (peak area)	1818	1862	1139	1432
Centrifugation 2: Contaminants remaining in product stream				
Condition	$Q/C\sum = 1.9 \times 10^{-9}$ m/s		$Q/C\sum = 1.7 \times 10^{-7}$ m/s	
Total protein (mg/mL)	0.26	0.26	0.38	0.41
Total lipid (peak area)	189.58	196.92	296.02	291.82

In comparing the "optimal" centrifugation strategy versus the "worst case" scenario for the pilot-scale experiments, it appears from **Table 7.2** that the former method was clearly more effective in eliminating yeast protein and lipid contaminants in the supernatant fraction during centrifugation 1 leading to a purer supernatant product stream in centrifugation 2. Product yield for HBsAg recovery would be dictated by the level of solids recovery from centrifugation 1 and the percentage dewatering from centrifugation 2. A greater level of solids loss during centrifugation 1 was observed for the "optimal" method due to the inefficiency of the scraper mechanism of the centrifuge in discharging solids which would impact product yield negatively if applied to the recombinant HBsAg process. The dewatering levels for centrifugation 2 appear comparable for both processes although the USD mimic predicts poorer performance for the "worst case" centrifugation.

7.4.4 Scale-up feasibility of the selective product recovery methodology

The scalability of the "optimal" strategy for the selective product recovery methodology was evaluated based on the USD and pilot-scale experiments with baker's yeast. Product yield for the recombinant HBsAg system would be dictated by the level of solids recovery from centrifugation 1 and the level of dewatering achieved in centrifugation 2. In this parallel study with baker's yeast, for centrifugation 1, a solids recovery level of ~ 96% was predicted from the USD mimic and based on the clarification performance at pilot-scale, a recovery level of ~ 90% would be achieved had solids been discharged effectively from the centrifuge. Although the pilot-scale data highlighted that solids loss due to the inefficiency of the scraper mechanism of the centrifuge in performing solids discharge could be an issue, it is likely that the magnitude of this problem would be less pronounced at industrial scale due due to the higher overall process volumes and throughputs. Both USD and pilot-scale data show high levels of dewatering of above 95% for centrifugation 2 which would ensure high HBsAg product recovery in the final supernatant stream. The results from the pilot-scale study also verified that the degree of purification afforded by the selective product recovery methodology would be maintained upon scale-up. Pilot-scale and USD data concur in that over 90% of host lipid and protein contaminants are eliminated in the supernatant stream during centrifugation 1 resulting in a significantly cleaner supernatant product stream in centrifugation 2.

Overall, the study with baker's yeast verifies that USD centrifugation experiments were reliable in predicting large scale centrifugation performance. The agreement of the USD and pilot-scale data in terms of the expected product yield based on the solids recovery characteristics and the degree of purification potentials afforded demonstrates that the benefits of the selective recovery methodology are not

compromised upon scale-up. Although the absolute values for these parameters may differ from system to system, it is reasonable to deduce from the baker's yeast data, which provides a parallel comparison to the recombinant HBsAg process, that the scale-up of the selective product recovery methodology would be equally feasible for the recombinant HBsAg material.

7.5 Conclusions

It is evident that the effectiveness of the selective product recovery methodology depends crucially on the performance of both key centrifugation operations. The value of USD techniques is clearly demonstrated in this study as it allowed a wide range of centrifugation operating conditions for HBsAg *S. cerevisiae* homogenate to be studied in a rapid fashion with millilitre volumes of material. The results show that for the first centrifugation operation, high $Q/C\sum$ conditions are favoured to ensure the majority of lipid and protein contaminants remain in the supernatant stream which is subsequently discarded. This however is provided that good solids recovery levels are achieved and HBsAg yields are not compromised. Since the HBsAg product is recovered in the supernatant fraction during the second centrifugation operation, good dewatering characteristics are vital for desirable product yields. For this, operating at the lower $Q/C\sum$ limit would be favourable. Pilot-scale studies performed using a parallel system with baker's yeast demonstrated that the USD mimics were indeed reliable in predicting larger scale centrifugation performance. Based on the high level of solids recovery and the effective reduction of host-derived contaminants observed from the USD and pilot-scale studies, we concluded that it would be feasible to scale-up the selective product

recovery methodology for commercial manufacturing of recombinant HBsAg. The optimal centrifugation conditions identified from the USD mimics is expected to be equally effective for the scaled-up process.

Chapter 8

Conclusions & recommendations for future work

8.1 Review of research objective & direction in view of issues in VLP development

Virus-like particles (VLPs) are an increasingly important class of vaccines which may hold the answer to the development of a host of preventative medicines against viral infections including HIV (Boisgerault *et al.*, 2002). An important breakthrough is in the development of chimeric and multivalent vaccines using VLPs as platforms for the presentation of foreign epitopes or targeting molecules (Grgacic and Anderson, 2006). This is especially key for disease conditions where in the natural state, recombinant surface proteins lack the spatial structure for eliciting a broad range of immune response.

In spite of the enormous potentials of VLP vaccines, the commercialisation of such vaccines is faced with immense challenges from the perspectives of stringent regulatory oversights, sophisticated product characterisation requirements, manufacturing considerations and scale-up issues (Buckland, 2005). Of these, the chief contributing factor to the slower reception and commercialisation of VLP vaccines than originally anticipated is the higher costs involved in the production and purification as compared to traditional life attenuated viral vaccines (Boisgerault *et al.*, 2002). Process development is particularly hampered by ineffective laboratory scale processes which

are difficult or costly to scale up and by a lack of process flexibility for improvements or modifications at later stages (Pattenden *et al.*, 2005). For commercial realisation of novel VLP vaccines, it is thus insufficient to focus research efforts on vaccine design and development. Instead, it is vital to complement such efforts with research in bioprocess manufacturing in order to develop effective and economical processes which can support the large-scale production and rapid delivery of the new vaccines to market.

In line with the issues highlighted above, the goal of this thesis is to develop a robust and enhanced primary purification route for future generation lipid-envelope VLPs in order to improve overall process efficiency and throughput. The focus on primary purification is especially relevant as early purification stages are often overlooked in process design. These operations could in reality be equally if not more important than high resolution operations as they often set the limits to the overall yield and levels of process performance that can be achieved.

It has been reported that chromatographic operations are the largest cost centre for downstream purification (Przybycien *et al.*, 2004). One of the means of driving down the reliance and purification burden on chromatography is by improving the efficiency of contamination reduction and product enrichment in the operations prior to chromatography. In this thesis, a novel approach for the redesign of primary purification processes was examined which integrated understandings of the VLP expression system and their physiochemical attributes with more conventional engineering methodologies and insights to process interaction. The three critical research milestones for this purpose include: (1) characterisation and optimisation of the conventional primary recovery route to improve VLP yield and antigenicity and to minimise the level of host-derived contaminants; (2) primary purification process re-design involving the introduction of an additional protein fractionation or separation step to improve

purification and product enrichment; and (3) scale-up considerations and feasibility assessment of the process developed from Stages 1 and 2. At all stages of process design, an equal emphasis was placed on maintaining the biological and physiochemical aspects of the HBsAg system as on purification performance which is vital from a regulatory perspective. Whenever possible, scaled-down bioprocess technologies, capable of predicting industrial scale performance, were applied. The low volume requirements and the shorter processing times using such scale-down mimics allow a wide matrix range of process variants to be evaluated in an effective, rapid and inexpensive way.

8.2 Summary of research findings

8.2.1 Characterisation & optimisation of detergent-mediated HBsAg liberation

Specific to the inherent expression characteristics of lipid-envelope VLPs in *S. cerevisiae*, product recovery is driven by a detergent step which facilitates the liberation of the VLP from the host organelle. Detergent-mediated VLP liberation is a critical step in primary recovery as it sets the framework for product yield, antigenicity and the profile of contaminants from the host cell which could impact downstream operations. The study with HBsAg demonstrates that the Triton X-100 detergent was the most effective agent for the extraction of the VLP from the host endoplasmic reticulum. The optimal concentration range for Triton X-100 was between 0.2 – 0.5% v/v. Concentrations below this range led to ineffective VLP recovery whereas concentration

above led to HBsAg delipidation which resulted in decreased VLP antigenicity. The level of co-liberation of protein and lipid contaminants into the product stream was in proportion to the amount of detergent employed. As a result, greater membrane resistance due to increased fouling effects was observed during ultrafiltration for samples generated using higher concentrations of detergent. Hence in general, a low detergent concentration is favourable for VLP liberation but the limiting factor in this would be the recovery level of active VLP.

8.2.2 Process improvement for primary purification

The first approach investigated involved the introduction of a protein fractionation step in primary purification via precipitation with polyethylene glycol (PEG) or ammonium sulphate. For HBsAg purification, PEG 8000 at a concentration of 10% w/v was found to be superior for the reduction of protein contaminants whilst ammonium sulphate at a concentration of 15% w/v was for lipid contaminants. In each case, an enrichment factor of ~ 8-fold was attained. The effectiveness of PEG precipitation was significantly influenced by the separation mechanism for precipitate recovery. Filtration was found to be superior to centrifugation when dealing with high viscosity process streams at PEG concentrations of 10% w/v and above. The main drawback with PEG and ammonium sulphate precipitations is in the modest step yields (50% - 70%) attained. In spite of this, the performance characteristics of the precipitation operations were sufficiently promising that, with further optimisation in terms of precipitation conditions and separation techniques, these operations may be practically and economically viable.

The second approach investigated for primary purification is derived from the exploitation of the intracellular compartmentalisation characteristics of lipid-envelope VLPs in *S. cerevisiae.* Due to the lack of protein transport machinery in recombinant yeast cells, the VLPs produced are permanently localised on cellular compartments such as the endoplasmic reticulum (ER) as in the case of the HBsAg. To impart selectivity to the product recovery process, a twin centrifugation strategy was proposed in which a centrifugation step is introduced immediately following cell disruption but prior to the addition of detergent for VLP liberation. In the HBsAg system investigated, the additional centrifugation step allowed the elimination of bulk cytosolic contaminants in the supernatant stream, achieving ~ 70% reduction of contaminating yeast proteins, lipids and nucleic acids. Recovery and subsequent treatment of the solids fraction with detergent released the HBsAg product into a significantly enriched product stream with a yield of over 80%. It was further demonstrated that the selectivity of this approach could be further enhanced by operating under moderate homogenisation pressure conditions (~ 400 bar) where improvements in the recovery of active HBsAg and greater reduction of contaminating lipids were observed. These were attributed to the lower shear conditions experienced by the HBsAg product and the reduced levels of cell fragmentation which led to lower co-liberation of lipid contaminants during the detergent step. The cleaner process stream resulted in a 40% improvement in product capture and a concomitant increase in step yield during a downstream hydrophobic chromatography operation. Although not evaluated in this study, the lower level of contaminants at the chromatography step is expected to improve column integrity and lifespan.

Overall, the selective product recovery approach is expected to be more effective than either of the precipitation methods using PEG or ammonium sulphate for

improving the performance of primary purification. This is essentially owing to the higher product yield (≥ 80%) achievable and its efficiency for the elimination of both yeast protein and lipid contaminant classes (≥70% reduction). In comparison, product yields from PEG and ammonium sulphate were in the range of 50% - 70% and their purification efficiencies were limited to proteins and lipids, respectively.

8.2.3 Scale-up considerations and assessment on process feasibility

Since the selective product recovery approach was deemed superior for primary purification, the scale up studies were focussed on this operation. The effectiveness of this strategy is dictated by the performance of each of centrifugation operation, specifically in terms of solids recovery, clarification, dewatering and the effective elimination of proteins and lipids contaminants. An ultra scale-down model was adopted in this study and the results demonstrated that the equivalent flow rate / settling area ($Q/C\sum$) parameters were critical in determining the effectiveness of the approach. Operating near the upper $Q/C\sum$ limit of the machine was found to be favourable for the first centrifugation step to ensure that the majority of host lipid and protein contaminants were discarded effectively in the supernatant waste stream and the low settling conditions did not affect adversely the level of solids recovery or HBsAg yield. For the second centrifugation operation, high centrifuge settling conditions at the lower $Q/C\sum$ limit of the centrifuge were desirable to achieve maximal dewatering characteristics to reduce product loss in the solids phase. Additionally, lower levels of protein and lipid contaminants were also measured in the supernatant product stream under these conditions. The scale-up studies using a parallel system with baker's yeast demonstrated that largely USD predictions of large scale performance were accurate.

The scale-up study also verified that the efficiency of the selective product recovery methodology in reducing the level of host-derived contaminants was not compromised by the increase in process scale. The single potential problem highlighted was related to the observation that some product loss occurred at pilot-scale due to the inefficiency of the centrifuge scraper mechanism in performing solids discharge. However, this problem is likely to be less pronounced at large scale manufacturing as the proportion of product loss would be minimal relative to the larger process volumes and throughputs experienced.

8.3 General conclusions

This study reaffirms the need for product characterisation to complement process design efforts. In the case of the HBsAg lipid-envelope product, the physiochemical and antigenic characteristics of the particle were shown to be sensitive to changes in the microenvironment for example in the level of detergent present in the process stream and the exposure of the particle to shear during homogenisation. In a commercial setting, extensive product characterisation is vital for the manufacturer to demonstrate complete assurance of the identity and composition of the vaccine and to prove equivalence between new and old processes if changes to the process route or scale were made (Buckland, 2005).

The influence of primary purification operations on downstream stages is evident from this study. For detergent-mediated HBsAg liberation, it was demonstrated the amount of detergent employed affected the level of co-liberated yeast protein and lipid contaminants which in turn affected the flux characteristics of a downstream ultrafiltration operation. In a similar study, the reduced level of contaminants, which

resulted from an improved primary purification operation, increased the binding capacity and throughput of a subsequent chromatographic operation. These findings emphasise the necessity for process design and development to be approached based on the bioprocess as an entirety rather than focussing on unit operations in isolation. Characterisation of process interaction is invaluable in identifying trade-offs to drive up overall process efficiency.

As in the case of many recombinant protein processes the separation potentials of primary purification operations for VLP production have yet to be fully realised. The re-examination of the primary purification process for HBsAg revealed that by the optimisation of detergent conditions for VLP liberation, the recovery of active product could be improved and the contamination profile reduced for the benefit of downstream operations. Alternative bioseparation technologies such as precipitation may provide an avenue for the reduction of processing burden and costs of chromatographic operations. The challenge, however, when additional processing steps are involved is in determining the level of acceptable compromise of product yield in favour of improved purification. For this purpose, it is vital that comprehensive economic feasibility studies accompany process development efforts.

This study has demonstrated how key understandings from cell biology can be implemented in the design of more effective purification methods for future generation VLP products. In the HBsAg model, the compartmentalisation of the VLP on the endoplasmic reticulum of the *S. cerevisiae* host was exploited to impart selectivity to the product recovery and purification process. This approach is increasingly pertinent with advances in biomolecular and cellular engineering which would enable the design of VLP expression systems to target the product of interest at specific sites of the cell for the ease of recovery and purification.

The future of VLP bioprocess design is integral with the use of effective scale-down technologies. This is particularly important to reduce ineffective process at laboratory scale which fail to deliver at manufacturing due to their inflexibility for scale-up and process modifications. Scale-down mimics of bioprocess technologies in contrast allow the assessment of process efficiency, robustness and scalability of industrial scale operations from the onset of process design. The use of such scale-down methodologies for ultrafiltration and centrifugation operations in the HBsAg study was invaluable in permitting a wide matrix of processing conditions to be investigated using small volumes of material in a rapid and cost-effective manner. The reliability of ultra scale-down centrifugation predictions were verified here by pilot-scale experiment whereas the predictability of the ultrafiltration scale-down model has been demonstrated elsewhere (Ma *et al.*, manuscript in preparation). A range of other scale down technologies for downstream processing are available and these can be incorporated routinely for more strategic VLP process development.

8.4 Recommendation for future work

The overall thesis objectives have focussed on the primary purification of lipid-envelope VLPs based on the HBsAg system. In so doing it has been possible to identify additional research areas which would be of interest as follow up studies to the primary purification work reported in the previous chapters.

8.4.1 Further analytical characterisation of HBsAg particles

Characterisation of HBsAg in this study relied heavily on ELISA assays which are based on antigenic activity measurements. The limitation of the method is that it does not provide indications of the actual conformation of the HBsAg particle. In certain cases, as discussed in **Chapter 4**, non-native HBsAg particles in the form of polypeptide micelles assembled under conditions of excess detergent can show antigenic activity measurements which are higher than the native particles. For more comprehensive particle characterisation, analytical techniques which probe for changes in structural properties of the HBsAg such as the use of circular dichroism (c.d.) and iodide quenching techniques (Gavilanes *et al.*, 1990) would be invaluable. Alternatively, optical imaging techniques such as confocal microscopy could be employed to obtain 3D images of the HBsAg particles. This technique is particularly valuable in permitting the visualisation of aggregation problems or HBsAg denaturation and irreversible adsorption of surfaces.

8.4.2 Further characterisation of HBsAg stability

The structural and physiochemical properties of VLP particles, due to their complex macromolecular structures, are highly influenced by their microenvironment and as aptly illustrated by Buckland (2005) the process of producing the VLP defines the product. The issue of HBsAg stability and antigenic activity has been broadly addressed in the study focussing on the impact of detergent condition during HBsAg liberation and the effects of varying homogenisation pressure conditions. However, other primary purification process parameters such as buffer pH and ionic strength, protein concentration, process and storage temperatures and the use of surfactants which

could impact VLP stability (Shi *et al.*, 2005) remain to be analysed. The use of microwell techniques (Aucamp *et al.*, 2005) combined with multi-factorial experimental design methods would be invaluable here to allow a large matrix of such processing conditions to be screened in a rapid and high throughput manner.

8.4.3 Optimisation studies to improve yield from precipitation operations

The levels of purification observed by precipitation using PEG and ammonium sulphate were promising however the problem lies with the low step yields attained. The poor yield from the PEG precipitation was attributed to the difficulties of precipitate resolubilisation and the loss of activity during this step. As discussed in **Chapter 5**, the structural stability of the HBsAg product can be improved by inducing HBsAg maturation through intraparticular disulphide bond formations which is typically accomplished only at the end of the purification process using thiocyanate and heat treatment (Zhao *et al.*, 2006). To promote HBsAg stability during precipitation, the possibility of operating the HBsAg maturation step prior to PEG precipitation should be investigated. This however is provided that the contaminants present do not interfere with the HBsAg oxidative refolding step and that the efficiency of subsequent purification processes are not affected.

In the case of ammonium sulphate precipitation, product loss was due to co-precipitation of HBsAg with lipid contaminants. Further studies to reduce the extent of such co-precipitation would be warranted and this may be achievable by optimising the conditions during precipitation such as the pH, ionic strength and the use of surfactants. The possibility of inducing HBsAg particle maturation prior to ammonium sulphate precipitation for increased HBsAg stability could also be explored.

8.4.4 Further characterisation of process interactions

As demonstrated in **Chapter 6**, the homogenisation conditions investigated have shown significant impacts on the recovery of active HBsAg and the level of lipid contaminants. In the HBsAg study, homogenisation pressure conditions were varied but other homogenisation parameters such as the number of passes and the design of the homogeniser such as the "impact" distance and the valve seat geometry were maintained as constants. Further studies should be extended to characterise these other parameters, in particular, the number of homogenisation passes which would have significant impact on the degree of cell breakage and the population size of the resulting cell debris. These factors would in turn affect the level of HBsAg recovery and the surface area available for co-extraction of membrane-derived lipids and proteins during the detergent step. These studies could be particularly valuable in further enhancing the efficiency of the selective product recovery methodology.

Significant levels of purification and product enrichment was attained using the selective recovery methodology. With the level of purification achieved using this primary purification strategy it may be possible to reduce the number of chromatographic operation downstream. To assess this, further studies would be required to gain better understanding of process interactions between primary purification and chromatographic stages.

8.4.5 Economic appraisal to complement process development efforts

Improvement in purification is usually at the expense of product yield as is apparent from the results in **Chapters 5-7**. Also, the development or modification of processes could have significant impacts on processing times and costs especially when

additional unit operations are incorporated. An economic evaluation is useful in such cases to set the boundaries for process development. Similarly, economic assessments should be extended to the HBsAg primary purification study to evaluate more comprehensively the overall benefits of the proposed processes. This is especially in line with the goal of developing processes which are practical and scalable for commercial manufacturing.

Chapter 9

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Appendix

Publication

Publication which arose from work discussed in **Chapter 4** on the development of a framework for detergent-mediated HBsAg liberation:

Kee, G.S., *Pujar, N., Titchener-Hooker, N.J., Study of detergent-mediated liberation of Hepatitis B virus-like particles from S. cerevisiae homogenate: Identifying a framework for the design of future-generation vaccine processes, Biotechnol. Prog. 2008, 24, 623-631 (doi: 10.1021/bp070472i)*